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In dieser Arbeit wird versucht, auf einige bei der Aufarbeitung der *in vitro* kultivierten Federanlagen beobachteten Einzelheiten hinzuweisen. Sowohl die Analyse von Federanlagen, die *in vitro* eine der *in vivo* vergleichbare Entwicklung aufweisen, als auch die von einigen davon weiter abweichenden Gebilden, erlauben gewisse Rückschlüsse auf die Vorgänge bei der Entstehung der Dunenfeder des Hühnchenembryos.

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6–35 mm² grosse Stücke Rückenhaut von 7–11 Tage bebrüteten Hühnchenembryonen wurden in der Regel nach der von Fell (2) angegebenen Uhrglasmethode, in einigen Fällen, insbesondere die Explantate von den älteren Embryonen, im Deckglas und in Carrelflaschen gezüchtet. Das Züchtungsmedium bestand entweder zu gleichen Teilen aus Hühnchenplasma (H. P.) und 50%igem Embryonalextrakt oder — zum kleineren Teil — aus 1,5 ml zur Hälfte mit Pannet-Compton'scher Salzlösung verdünntem H. P., dem 1 Tropfen konz. Embryonalextrakt zugesetzt war. Die Explantate wurden bei 38,5° C bebrütet, zum ersten Mal nach 48^h–72^h, dann in Abständen von 48^h auf neues Medium umgesetzt. Die längste Kulturdauer betrug 168 Stunden.

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Da die Stückchen Rückenhaut in der Regel vollständig auf dem Züchtungsmedium ausgebreitet waren, liess sich immer wieder leicht ihre vorherige Lage im Spenderembryo rekonstruieren; demnach beziehen sich die verwendeten Ausdrücke, wie „caudal", „cranial" u. dgl. auf den Spenderembryo.

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Davies (1) hat 1889 die Entwicklung der Dunenfedern sehr eingehend beschrieben. Späterhin haben u. a. Watterson (9) und Goff (4) einzelne Vorgänge dieser Entwicklung näher untersucht, ersterer die Bildung der Rami und die Rolle der Melanocyten, der zweite die Rolle des Mesenchyms. So ist es als Grundlage für die Betrachtungen *in vitro* hier nur notwendig eine grosse Linie anzudeuten. Allein einige Beobachtungen in der frühesten Entwicklung der Dunenfeder, die von der ausführlichen Darstellung Davies' abweichen, mögen besonders erwähnt werden. Es werden nur die Dunenfedern der dorsalen Federflur betrachtet.

Die ersten Anzeichen ihrer Entstehung sind etwa nach 7 ½ bis 8 ½ Be-brütungstagen zu finden. Es handelt sich um eine primitive dermo-epidermale Anlage. Histologisch lässt sich Folgendes erkennen: Vor dem Erscheinen der ersten Anlagen besteht die Epidermis aus einer meist einschichtigen Lage von Zellen. Ihre oft ovalen Kerne liegen parallel zur Oberfläche. Diese Schicht ist von einer Lage sehr platter Zellen, dem Periderm bedeckt. Im dermalen Bindegewebe ist eine dichtere, dem Epithel direkt unterliegende Zone, und eine breite Schicht sehr lockeren Gewebes zu unterscheiden. An ihrer Grenze befindet sich ein dichtes Kapillarnetz. Zu Beginn der Federentwicklung entstehen dicht unterhalb der Epidermis in regelmässigen Abständen Verdichtungsherde mesenchymaler Zellen. Die darüberliegenden scheibenförmigen Epidermisabschnitte werden höher. Ihre Zellen ordnen sich senkrecht zur Oberfläche an und die Epidermislage selbst wird 2-, in dem caudalen Sektor 3-schichtig. Denn jetzt bereits zeigt sich eine radiäre Asymmetrie der Federanlagen, die wenig später zu der caudalen Wachstumsrichtung des Dunenfederkeimes führt. In der caudalen Hälfte der scheibenförmigen Epithelverdickung erreichen darüber hinaus die Zellen eine besonders hoch zylindrische Form. Ihre Kerne liegen weit apical und lassen basal einen breiten Saum kernfreien Cytoplasmas. Die Peridermzellen sind im ganzen Bezirk der Federanlage höher, dies ebenfalls wiederum im caudalen Abschnitt in besonderem Masse. Abb. 1 zeigt ein Stadium, in dem die Bildung dieser primitiven Federanlage bereits zum Abschluss gelangt ist. Durch ein starkes Wachstum der gesamten Papille hebt sich der Federkeim in Form eines vorn leicht abgerundeten Konus über das Niveau der Haut hinaus. Dabei bleibt zunächst die caudale Hälfte des Epidermismantels höher als die craniale. Sie führt sozusagen das Wachstum der Federknospe an. Ihr Mittelpunkt wird dann zur Spitze des zylindrischen Federkeimes. Davies (1) glaubte, dass die caudale Wachstumsrichtung durch eine stärkere Proliferation am cranialen Umfang der Anlage bedingt sei und auch Goff (4) z.B. übernimmt diese Annahme. Demgegenüber lassen sich unsere Beobachtungen nur durch ein aktives Wachstum der ganzen Anlage in caudaler Richtung erklären. Es findet auch nicht nur eine Zellvermehrung an der Basis des Epidermismantels der Federanlage statt, wie es Davies feststellte. In dem ganzen Epidermismantel, besonders aber auch in den apicalen Abschnitten lassen sich Mitosen erkennen. Der Längsschnitt der Abb. 4 zeigt den Aufbau des zylinderförmigen Federkeimes etwa am 10.–11. Tage. Es handelt sich zwar um Federknospen, die *in vitro* entstanden sind. Sie entsprechen jedoch ganz dem, was man *in vivo* antrifft, mit der Einschränkung, dass die Pulpa hier keine Blutgefässe aufweist.

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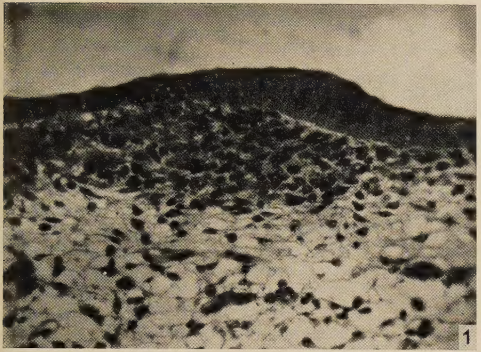
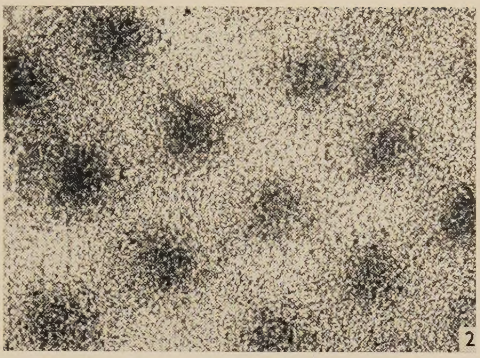
Anteils der Feder die Entstehung von Längsleisten, den späteren Rami zu beobachten. Sie beginnt distal und schreitet nach proximal fort. Während sie auf dem Längsschnitt als Säulen erscheinen, springen sie auf einem Querschnitt bogenförmig gegen die Pulpa vor (vgl. Abb. 5, die wiederum von einer Kultur herrührt, aber den normalen Aufbau gut wiedergibt). Die einzelnen Schichten sind wie folgt an diesem Vorgang beteiligt: Das Periderm bildet eine platte, einschichtige und verhornte, äusserste Lage. Die äusseren Lagen der Intermediärschicht der Epidermis verlaufen zirkulär und bilden die ebenfalls früh verhornende Federscheide. Die tiefer liegenden Zellen der Intermediärschicht ordnen sich zu den Zellsäulen der späteren Rami an, während sie von der zunächst noch einschichtigen Basallage umschlossen und gegen die Pulpa abgegrenzt werden. Auch alle weiteren Veränderungen beginnen distal und schreiten proximal fort. Die Zellsäulen, die sich in sich wieder weiter differenzieren (vgl. z.B. Watterson (9)) und verhornen, werden von der Basalschicht immer vollständiger umschlossen und so auch von der Federscheide abgegrenzt. Das Gewebe der Pulpa das zunächst aus sehr dicht gedrängten Zellen bestand, wird bis auf einen proximalen Abschnitt immer lockerer. Schliesslich ist es nur noch ein Netzwerk fein verästelter Zellen. Als Ganzes bleibt die Pulpa hinter dem Wachstum der Zellsäulen zurück, sodass sie sich von diesen ablöst. Dabei bildet die Basalschicht, die mehrschichtig geworden ist und sich längs spaltet, eine die Pulpa einhüllende Membran und die Membranen der Rami. Bei der Rückbildung der Pulpa kommt es dann durch Verhornungsvorgänge an der Pulpamembran zur stufenweisen Bildung von Hornkappen. Diese sind in ihrem Zentrum untereinander durch Hornfäden verbunden (vgl. Goff (4)).

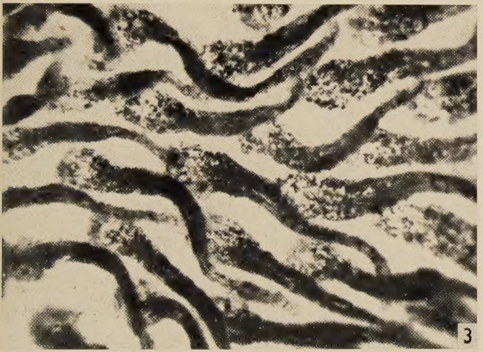
An der Basis der Federknospe hat inzwischen um den 11.–12. Bebrütungstag die Ausbildung eines eigentlichen Federfollikels begonnen. Dabei wird die Basis der Feder unter das Niveau der Haut versenkt. Davies (1) hat darauf hingewiesen, dass es sich dabei um einen aktiven Wachstumsvorgang in die Tiefe, an dem alle Schichten der Federbasis ausser dem Periderm teilnehmen, handelt.

BEOBACHTUNGEN AN *IN VITRO* GEZÜCHTETEN FEDERANLAGEN

In vitro wurden im Wesentlichen dreierlei Wege des Wachstums und der Differenzierung der Federanlagen beobachtet. Welchen davon das Explantat einschlägt, hängt in erster Linie von dem Bebrütungsalter des für das Explantat verwendeten Embryos ab. Eine grosse Rolle spielt die Züchtungsart d.h. ob im Uhrglas nach der Methode von Fell (2), in der Carrelflasche







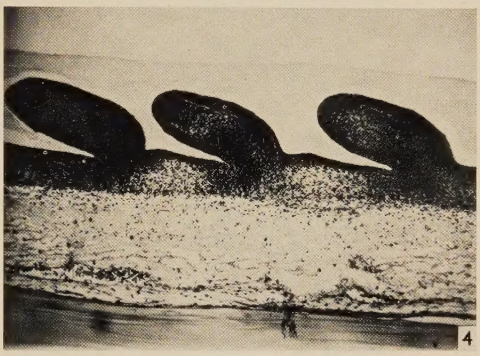


Abb. 1. Primitive dermo-epidermale Federanlage. Rückenhaut Hühnchenembryo, 8. Bebrütungstag. Stieve-Hämalaun, 380 ×.

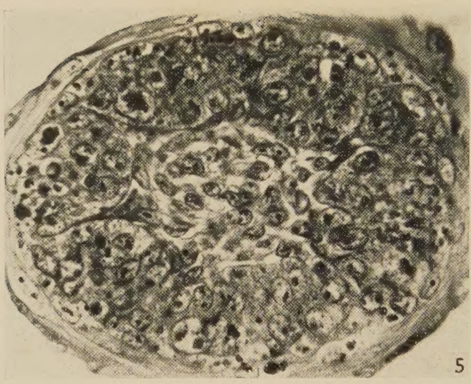
Abb. 2. Primitive dermo-epidermale Federanlage. Rückenhaut Hühnchenembryo, 8. Bebrütungstag, frisch explantiert, Lebendpräparat, 90 ×.

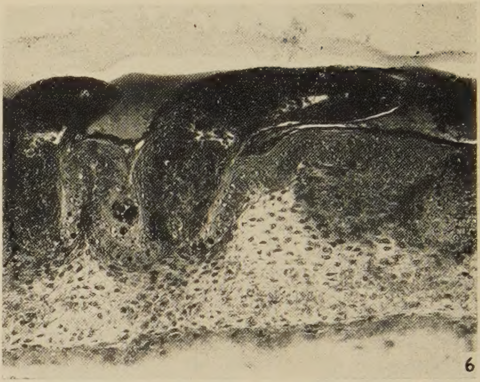
Abb. 3. Rückenhaut im Stadium der Abb. 2 entsprechend explantiert. 168 Stdn *in vitro*. Uhrglasmethode, Lebendpräparat, 90 ×.

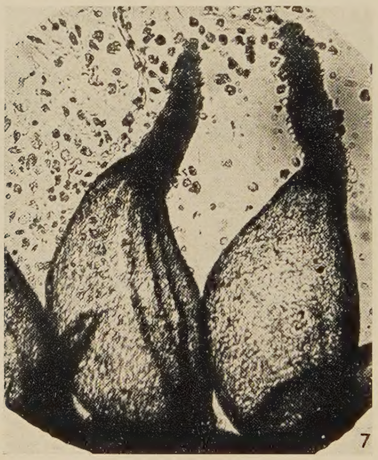
Abb. 4. Zylinderförmige Federknospen, im Stadium der Abb. 1 und 2 entsprechend explantiert. 50 Stdn *in vitro*. Stieve-Masson, 90 ×.

oder im Deckglas, sodann ob in einem extraktreichen oder einem extraktärmeren Medium gezüchtet wird. Andere Faktoren, wie z.B. das Alter der zum Extrakt verwendeten Embryonen (3), Züchtung auf durchlochtem Zellophan nach Earle als fester Unterlage, u.s.w., waren wirkungslos.

Eine erste Beobachtungsreihe bezieht sich auf die Explantation von Hautstücken mit Federanlagen im Stadium der primitiven dermo-epidermalen Anlage (Abb. 2). Hier vollzieht sich eine Organentwicklung, die bis zu 7 Tagen *in vitro* in vielen Punkten die normale Federentwicklung imitiert und vor allem in der ersten Zeit mit ihr auch zeitlich Schritt hält. — In einer zweiten Gruppe lassen sich die Ergebnisse mit der Explantation von Rückenhäuten, z.B. von 7 Tage bebrüteten Hühnchenembryonen, die noch keine







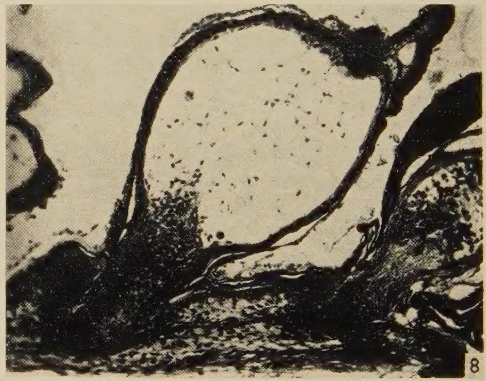


Abb. 5. Querschnitt durch den distalen Abschnitt einer Federknospe im Stadium der Abb. 1 und 2 entsprechend explantiert, nach 72 Stdn *in vitro*. Stieve-Masson, Öl, 800 ×.

Abb. 6. Gleiche Kultur wie Abb. 3, vgl. auch mit Abb. 1. Stieve-Masson, 180 ×.

Abb. 7. Ballonförmig aufgetriebene Federknospe. Explantat von einem 11 Tage bebrüteten Embryo, 44 Stdn *in vitro*. Carrelflasche, Lebendpräparat, 50 ×.

Abb. 8. Ballonförmig aufgetriebene Federknospe. Explantat von einem 10 Tage bebrüteten Embryo, 54 Stdn *in vitro*. Stieve-Eisenhämatoxylin, 180 ×.

sichtbaren Federanlagen aufweisen, zusammenfassen. Hier entstehen meist unförmige, missgebildete und der charakteristischen Rückwärtsneigung ermangelnde Federknospen. — Drittens endlich liess sich beobachten, dass Federknospen, die sich vor der Explantation bereits über das Stadium der primitiven Anlage hinaus entwickelt hatten, in zunehmendem Masse *in vitro* ihre Differenzierungsfähigkeit verloren und, sofern sie nicht zugrunde gingen, sich sehr häufig in ein gestieltes, kugeliges bis ballonförmiges Gebilde, wie es die Abb. 7 zeigt, umwandelt.

Diese drei Beobachtungsgruppen sollen in derselben Reihenfolge etwas näher betrachtet werden.

1) Das Anfangsstadium und das Endergebnis nach 7-tägiger Kultur zeigen die Abb. 2 bzw. 3. Der histologische Aufbau der primitiven Federpapille ist bereits Seite 293 beschrieben. Bei Verwendung dieses Stadiums als Ausgangspunkt lässt sich nach 24 Stunden *in vitro* eine konusförmige, über das Niveau der Haut herausragende Anlage erkennen. Sie ist deutlich nach caudal gerichtet. Dieser Umstand wird besonders dadurch unterstrichen, dass sich der Punkt der grössten Höhe des Epidermismantels unterhalb der Spitze, auf dem caudalen Umfang der Anlage befindet. Nach 48 Stunden bietet die Federknospe die Form eines Zylinders. Abb. 4 zeigt ihren Bau zu diesem Zeitpunkt. Der epidermale Mantel ist nun in den oberen Teilen der Knospe gleichmässig dick, während er sich an der Basis verdünnt. Mitosen sind über den ganzen Mantel verteilt. Es lässt sich nicht erkennen, dass an dem Übergang von der schmalen zu der breiteren Zone ein Ort erhöhter Proliferation ist, so wie es Davies (1) berichtet. Nicht selten ist die äusserste Schicht, das Periderm, sehr hoch oder gar mehrschichtig. Sie kann eine regelrechte Kappe, die dem Federkeim aufsitzt, bilden. Dabei handelt es sich um ein Pseudo-Längenwachstum der Federanlage, das Rossi und Borghese (8) beschrieben. Das Periderm der Feder, in dem sich ebenfalls zahlreiche Mitosen beobachten lassen, neigt demnach unter den Bedingungen *in vitro* zu einer begrenzten, aber selbstständigen Proliferation. Dagegen bleibt das Periderm der restlichen Haut einschichtig und bildet wie *in vivo* eine früh verhornende, dünne Lamelle. Die Pulpa ist meist aus dichtgedrängten, gleichförmigen, mesenchymalen Zellen zusammengesetzt. Mitosen sind häufig zu sehen. Gelegentlich finden sich im Zentrum der Pulpa ein oder zwei Kapillaren, die mit dem zum Zeitpunkt der Explantation schon vorhandenen Kapillarnetz des Derma in Verbindung stehen. Diese Kapillarsprossen haben sich demnach *in vitro* gebildet, obwohl eine Blutversorgung nicht mehr bestand.

In der Folge, etwa von der 72. Stunde ab, lassen sich zwei weitere Vorgänge beobachten: In den apicalen Bezirken der Pulpa beginnend findet eine Auflockerung der dicht gedrängten Zellen statt. Dies entspricht weitgehend den Verhältnissen *in vivo*. Andererseits gliedert sich der Epidermismantel in eine Basal-, eine Intermediärschicht und die Federscheide. Während nun aber *in vivo* aus der geordneten Proliferation der Basal- und Intermediärschicht die Zellsäulen der späteren Rami hervorgehen, wird dieser Prozess *in vitro* nur in einer sehr unvollständigen Weise angedeutet. Doch die Ansätze lassen sich immer wieder erkennen. In einigen wenigen Fällen wird sogar eine Differenzierung erreicht, die der normalen ganz entspricht (vgl. Abb. 5). Meist lassen sich jedoch nur einige unregelmässige Verdikkungen der Intermediärschicht, die dann oft wieder verschwinden, erkennen.

Die weitere Entwicklung *in vitro* führt — soweit es die Lebendbeobachtung erkennen liess — vor allem zu einer Zunahme an Länge und Schlankheit der Dunenfederknospen. Abb. 3 veranschaulicht ihr endgültiges Aussehen, so wie es die angewandte Methode zu erreichen gestattet. Öfters jedoch sieht man gegen Ende der Züchtungsperiode, dass sich die Spitze der Dunenfederknospe leicht aufbläht. Auf dem Schnitt besteht dann dieser Abschnitt aus einer 1–2schichtigen Lage der intermediären Zellschicht und der Federscheide, die einen Raum, in dem die Pulpazellen nur ein äusserst lockeres Netz bilden, umschliessen.

Ein histologisches Bild, das der Abb. 3 entspricht, zeigt die Abb. 6. Man erkennt in dem mittleren Teil des Schafts (d.h. fast des ganzen über der Haut liegenden Federanteils — wegen der Krümmung des Schafts ist nur ein Teil in der Schnittebene) eine Andeutung von Rami-Anlagen. Sie bestehen jedoch statt aus verhornten Zellsäulen aus etwas unregelmässigen, gleichfalls verhornten Zellplatten. Der Federschaft ist von der verhornten Federscheide eingeschlossen. Das Periderm dagegen, das normalerweise eine dünne Hornmembran bildet, ist weiter proliferiert und sitzt gelegentlich in hohen Lagen oder Kappen den Federn auf. Die Pulpa ist sehr locker. Auch hier bildet sie nur noch ein loses Netzwerk fein verästelter Zellen.

Auf dieser Abb. 6 erkennt man einen wesentlichen Teilprozess der Federentwicklung, der sich der Lebendbeobachtung entzieht, auf den Schnitten aber regelmässig zu beobachten ist. Die Basis des Federkeims hat sich unter Bildung eines Follikels unter die Haut eingesenkt. Dieser Vorgang vollzieht sich zwischen dem 4. und 5. Kultivierungstag. Man findet daran alle Details, die auch bei der Follikelbildung *in vivo* vorhanden sind, wieder. An der Einsenkung in die Tiefe haben alle Schichten, mit Ausnahme des Periderms, teilgenommen. Die unteren Lippen der epithelialen Wandung bilden den Umbilicus. Die Pulpa der Federbasis ist im Gegensatz zu den distalen Abschnitten dicht. Im Ganzen fällt bei der Betrachtung der Federknospen in einem solchen Stadium auf — und die Abbildung lässt es erkennen — dass die Höhe des Differenzierungsgrades proximal am höchsten ist und nach distal zu abnimmt.

Bei diesem Entwicklungsweg ist ein erster Abschnitt des Wachstums der Federknospe von ganz besonderer Regelmässigkeit, nämlich der, der dem Weg von Abb. 1 bis Abb. 4 entspricht (5). Dabei vollzieht sich einmal ein gerichtetes Wachstum des epidermalen Anteils, in dem Sinne wie es S. 293

beschrieben wurde, sodann eine starke Proliferation von Pulpazellen: zwei Vorgänge die durch ihren parallelen Ablauf das organgemässe Wachstum bedingen.

2) Es zeigt sich, dass bei der 2. erwähnten Gruppe von Beobachtungen, nämlich denen an Explantaten, die noch keine primäre Federanlage aufweisen, die oben gemachte Feststellung nicht zutrifft. Es fehlt diesen Federknospen vor allem die Eigenschaft des räumlich ausgerichteten Wachstums. Im epidermalen Mantel ist keine Bevorzugung der Proliferation in einem caudalen Sektor der scheibenförmigen primären Anlage, die erst *in vitro* entsteht, zu erkennen. Es handelt sich vielmehr nur darum, dass das Wachstum der bindegewebigen Papille samt der ihr aufliegenden Epidermis zu einem Hervordrängen über das Niveau der Haut führt. Entsprechend sind die dabei beobachteten Formen oft von plumpem Aussehen. Auffallend allein ist die Häufigkeit von Mehrfachbildungen d. h. Äquivalenten von Federknospen, die sich in 2–3 das Niveau der Haut überragende Stümpfe von gleicher oder unterschiedlicher Grösse gabeln.

3) Es ist bereits erwähnt worden, dass gegen Ende der Züchtung *in vitro* oft eine Auftreibung der Spitze der Dunenfederknospe entstand. Waren zur Explantation bereits gut individualisierte Federkeime, etwa dem Stadium der zylinderförmigen Knospe (vgl. Abb. 4) entsprechend, verwendet worden, so liess sich kein weiteres Wachstum erkennen, vielmehr trat eine Umwandlung der Knospe als Ganzes in ein ballonförmiges sehr zartwandiges Gebilde, das einem Stiel aufsitzt (Abb. 7), ein. Bei Züchtung in der Carrelflasche, im hängenden Tropfen, mit dem extraktärmeren der beiden verwendeten Medien und besonders bei Aufrechterhaltung einer reinen O_2-Atmosphäre erschienen diese Ballons besonders stark aufgetrieben.

Abb. 8 stellt einen Schnitt durch ein solches dar. Es handelt sich im Prinzip um denselben Vorgang, der oben bereits erwähnt wurde. Man erkennt, dass die zu Beginn der Explantation meist schon vorhandenen Anlagen der Zellsäulen vollständig verschwunden sind. Die Wandung besteht aus einer Basalschicht und der verhornten Federscheide. Die Pulpa ist im grössten Teil des Gebildes ein sehr lockeres Netzwerk fein verzweigter Zellen, proximal jedoch immer noch aus dicht gedrängten Zellen bestehend. Die beiden Pulpa-Abschnitte sind scharf abgegrenzt. Abb. 8 zeigt, dass ein weiterer Differenzierungsvorgang, der hier vollständig aus seiner normalen Beziehung herausgelöst ist, stattgefunden hat. An der Spitze der Knospe hat sich die Basalschicht gespalten und bildet eine Pulpamembran. Unter Resorption der Pulpa entstehen deutlich erkennbare Hornkappen, von denen hier 2 zu sehen sind.

Innerhalb der epidermalen Anteile der Federanlagen von Embryonen pigmentierter Rassen sind, wie bei Lebendbeobachtung auch bei der histologischen Untersuchung melaninhaltige, verzweigte Melanocyten und melanintragende Epidermiszellen zu erkennen (vgl. Abb. 6).

BESPRECHUNG

Wenn diese Befunde zusammengefasst werden sollen, so müsste dies unter einer Voraussetzung geschehen: Zu dem Zeitpunkt, in dem die Rückenhaut des Hühnchenembryos explantiert wurde, dürften die Anlagen der Dunenfeder bereits vollständig determiniert sein, sind es auf jeden Fall bei den Explantaten von älteren Embryonen. Die *in vitro* zu beobachtende Entwicklung stellt nur den autonomen Ablauf bereits determinierter Differenzierungsvorgänge dar. Die Mängel dieser Entwicklung lassen sich daher auf eine Insuffizienz des sich differenzierenden Substrates, der Zelle, zurückführen (10). Diese Insuffizienz ist die Folge des künstlichen Milieus, das die Kultur *in vitro* bietet.

Drei Beobachtungen bei der Entwicklung der Dunenfeder *in vitro* sind es, die im Vergleich mit ihrer Entwicklung *in vivo* von Interesse sind. 1) die mangelhafte Bildung der primären Anlagen *in vitro*, 2) die mangelhafte Bildung der Zellsäulen und 3) die Entstehung der ballonähnlich aufgetriebenen Federknospen.

Wohl können Rückenhäute, die zum Zeitpunkt der Explantation noch keine primären Anlagen besitzen, solche bilden. Doch diese erweisen sich für eine weitere organotypische Differenzierung als unfähig. Es entstehen Federknospen unterschiedlicher Grösse und Form, Mehrfachbildungen und vor allem fehlt die charakteristische Ausrichtung des Wachstums. Kurz, es handelt sich zwar um ein koordiniertes Wachstum des dermalen und epidermalen Anteils der Federanlage, doch zugleich um ein ungerichtetes, nicht organgemässes Wachstum, eben vorwiegend ein Wachstum, während die organgerechte Differenzierung fehlt oder nahezu fehlt. Inwieweit hier dieser Mangel auf den Zustand des epidermalen Anteils zurückzuführen ist, mag dahingestellt bleiben.

Der andere wesentliche Differenzierungsschritt, der *in vitro* meist nur angedeutet wird, ist die Entstehung der Zellsäulen, der späteren Feder-Rami aus der Matrix der intermediären Zellschicht des epidermalen Mantels der Federknospe. Ansätze zu der lokalen Verbreiterung innerhalb dieser Schicht und zu der *in vivo* zu findenden Ausrichtung der daran teilnehmenden Zellen sind zu beobachten. Dabei ist es interessant zu sehen, dass diese

Ansätze besonders in den proximalen Abschnitten der Federknospe gemacht werden. Unter den Bedingungen der Kultur *in vitro* sind die Ernährungsverhältnisse dort gewiss besser, als in den distalen Abschnitten, die sich ja während der Entwicklung *in vitro* zunehmend von dem ernährenden Kulturmedium entfernen (vgl. auch S. 298).

Gebilde endlich, wie sie die Abb. 7 u. 8 zeigen, stehen offensichtlich mit der mangelhaften Differenzierung der Zellsäulen in Zusammenhang. S. 299 wurde darauf hingewiesen, dass zugleich mit der Streckung der Zellsäulen eine Auflockerung der Pulpa stattfindet. Diese Auflockerung führt zu dem weitmaschigen, losen Netzwerk der Pulpa in den distalen Bezirken der Federknospen. Die Streckung der Zellsäulen, die den Pulparaum vergrössert, fehlt jedoch. Andererseits, und das betrifft besonders die Explantate von älteren Embryonen, kommt eine frühzeitige Verhornung der äussersten Epidermisschichten der Federknospe hinzu. So nimmt die ganze Knospe, bzw. der betroffene Bezirk schon aus mechanischen Gründen eine Kugelform an. Zwei Prozesse, Auflockerung der Pulpa und Streckung der Zellsäulen, die *in vivo* koordiniert sind und von denen z.B. Davies annahm, dass der erstere die mechanische Folge des zweiten ist, lassen sich so *in vitro* dissoziieren. Dies mag ein Beispiel dafür sein, dass ein Differenzierungsvorgang, infolge des Fehlens eines anderen ihm zugeordneten, autonom werden und so zu einer Missbildung führen kann.

ZUSAMMENFASSUNG

Bei der Entwicklung von Dunenfederanlagen *in vitro* wird eine organgemässe Entwicklung beobachtet. Eine intakte primäre Anlage ist dazu Voraussetzung. Wird sie erst *in vitro* gebildet, so führt sie nur zu koordiniertem Wachstum der aufbauenden Gewebe, Epidermis und Mesenchym, ohne eine organgemässe Differenzierung. Die Differenzierungsfähigkeit nimmt infolge der besonderen Ernährungsbedingungen in der Kultur von proximal nach distal ab. Da es sich wahrscheinlich um bereits determinierte Differenzierungsvorgänge handelt, sind Mängel der Entwicklung *in vitro* auf eine — eventuell ernährungsbedingte — Insuffizienz der sich differenzierenden Substrate, den Zellen, zurückzuführen. Aus einem Beispiel der Dissoziation zweier *in vivo* koordinierter Prozesse wird auf die Autonomie der einzelnen Differenzierungsvorgange geschlossen.

Teilabschnitte der hier beschriebenen Entwicklung lassen sich zur methodischen Untersuchung der Einwirkung äusserer Faktoren auf das organotypische Wachstum verwenden (5).

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A MICRORESPIROMETER FOR SIMULTANEOUS MEASUREMENT OF O_2-CONSUMPTION AND CO_2-PRODUCTION AND ITS USE IN DETERMINING THE RESPIRATORY QUOTIENT OF TISSUE CULTURES

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A microrespirometer was devised for the purpose of making accurate measurements of the Respiratory Quotient (R. Q.) of tissue cultures, but it is suitable for any kind of microrespiration work.

It is based on the combination and improvement of two techniques already known: 1. O_2-consumption being measured by a micromodification of Warburg's constant-volume method according to the principle described by Gregg (8); 2. CO_2-production by the change in electric conductivity of a CO_2-absorbing $Ba(OH)_2$-solution, a method introduced into microrespirometry by Fenn (5) and von Ledebur (12).

Volumetric measurement of O_2-consumption

The Gregg method was modified in the following way:

The measuring system was made independent of changes in atmospheric pressure by connecting the open limb of the manometer system with a relatively "infinitely large" compensating vessel V_c (Fig. 1), submerged in the same water bath as the measuring vessel V_m (10).

The form of the measuring vessels was modified for our purposes.

Sufficient sensitivity was obtained by:

1) the greatest possible reduction in vessel volume. Limitations are imposed by the dimensions of the respirating objects and by the difficulty of handling too small vessels.

2) the use of an inclined manometer. Sensitivity of such a manometer depends on the angle of inclination.

Construction of the volumetric part. The measuring vessel V_m (Figs. 2, 3 and 4) is connected to a piece of capillary tube—about 20 cm long, inner diameter about 0,3 mm—by means of a flange joint. The *polished* (not merely ground) surfaces of this joint require a *minimal amount* of grease for tightness. *This is essential for good operation.*

If the surfaces are ground with emery only, the grease layer must be made so thick that it is subject to deformation. This causes considerable changes in vessel volume during measurement.

The upper dilated part of the capillary ends in a 6 mm conical standard joint *J* (Fig. 3), by means of which the *unit*, consisting of measuring vessel and capillary assembled together, can be connected to the manometer system. The dilated part of *J* facilitates filling of the capillary with an index fluid as will be shown below.

Several units can be placed in a line in the water bath, each in a clamp as shown in Fig. 3. This clamp can be moved vertically by means of key *K* in order to bring the index meniscus *i* into the field of the travelling microscope *M*. As after the preliminary setting the unit should remain in the same position during the experiment, the clamp can be locked by means of screw *L*.

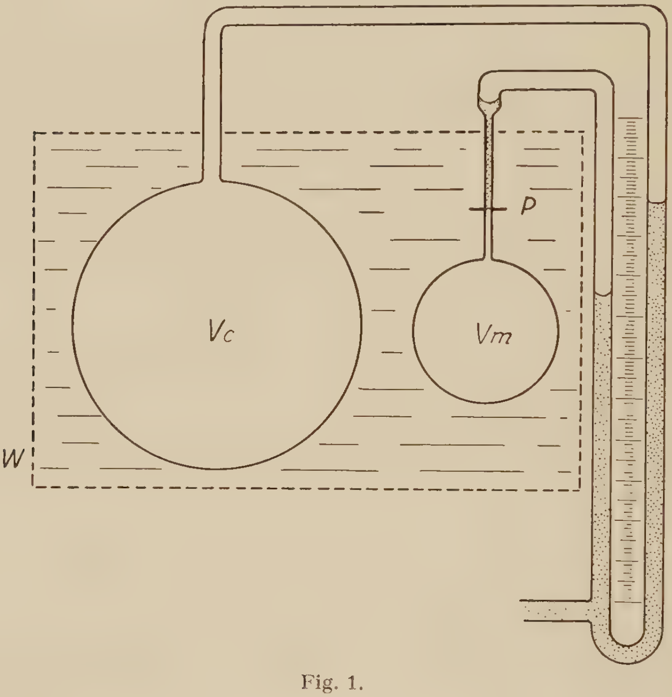

Fig. 1.

The microscope can be moved along the row of measuring units over a horizontal rail *R*, which ensures its return on the same level in front of each capillary. It is provided with a crosshair in its eyepiece. Adjustment of the constant volume is made by adjusting the index meniscus to this hair (Fig. 2, detail underneath *M*).

A narrow white strip *O* behind each capillary presents an even background against which the index meniscus stands out clearly.

In constructing the inclined manometer attention should be paid to the following points:

Accuracy in indication is influenced by differences in diameter along the narrow tubes used (capillary forces!) and by slight divergences from the straight line of each of the manometer limbs and from their parallelism. These handicaps can be avoided by using constant-bore tubes and by supporting them with care. Such a manometer works very satisfactorily, having a sensitivity e.g. ten times as great as a vertical manometer, and it can be read just as easily.

The tube bore should not exceed 3 mm, for if it does, deformation of the fluid menisci by gravity will impair accurate reading.

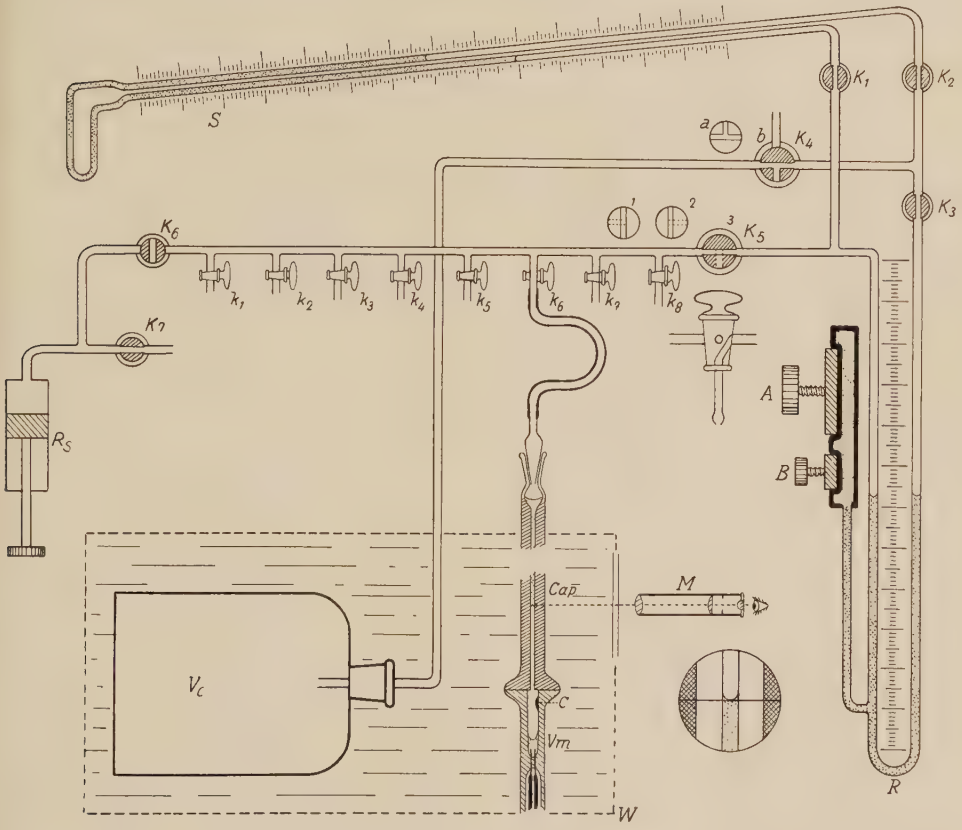

Fig. 2. Schematic diagram of the microrespirometer.
S = inclined manometer. R = vertical manometer. A and B = screws for coarse and fine adjustment of the pressure in the respirometer respectively. M = microscope, underneath M the picture of the capillary and its index meniscus as seen in the eyepiece of the microscope are shown. R_s = syringe 20 cc. Cap. = capillary. V_m = measuring vessel. C = respirating material. V_c = compensating vessel, a 5 liter flask. W = water bath. K and k mean stopcocks, underneath K_5 its special type is represented.

Brodie's solution is used as a manometer fluid.

Measuring procedure. The measuring vessel, after being thoroughly cleaned with concentrated HNO_3 for at least 24 hours, is rinsed with distilled water several times and *dried* and *sterilized* by several washings of *freshly distilled* acetone.

If not freshly distilled, the acetone will often on drying leave behind some greasy substance, which is easily oxidized, especially when coming into contact with alkali ($Ba(OH)_2$!).

A quantity of $Ba(OH)_2$-solution is pipetted onto the bottom of the vessel (see also page 310). The respiration material is made to adhere to the vessel wall at some distance of the $Ba(OH)_2$.

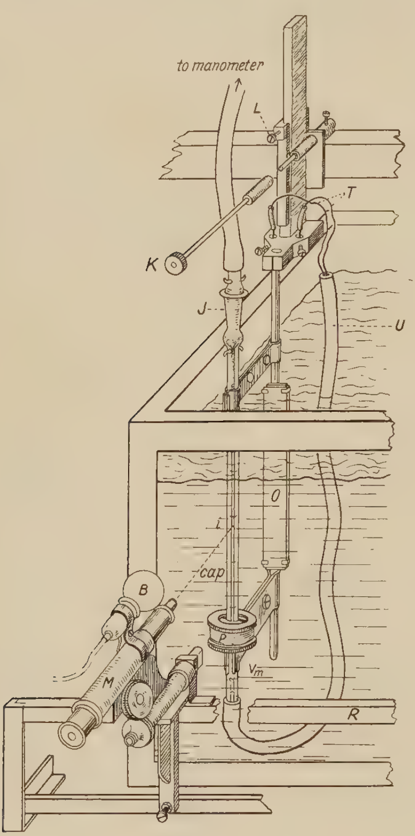

Fig. 3.

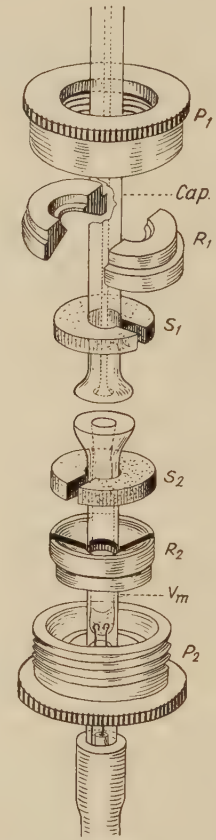

Fig. 4.

Fig. 3 (left). View of measuring unit, clamp and sliding microscope. L = screw locking the vertical movement of the clamp. K = key controlling the vertical movement of the clamp. T = electric leads from the electrodes of the measuring vessel to the Wheatstone-bridge circuit. U = flexible tube insulating the leads T from the water of the bath. O = white strip, forming the clear background necessary for observation of the index meniscus *i* in the capillary *Cap.* by means of the sliding microscope M. R = rail along which the microscope M slides along the row of capillaries. J = standard joint connecting the unit to the manometric system. B = electric bulb illuminating the index meniscus.

Fig. 4 (right) "Exploded" view of the mechanism by which the capillary and measuring vessel are united. P_1 and P_2 = brass rings that are screwed into each other. R_1 and R_2 = half rings of brass transferring the pressure from P_1 and P_2 to the rubber rings S. Cap. = capillary with flange joint. V_m = measuring vessel with flange joint.

A minimal amount of grease[1] is then spread on to the polished joint surface and the capillary is attached to the vessel in the way indicated by Figs. 4 and 5. Thereafter, the assembled unit is placed in the water bath and temperature equilibration can take place. The expanding air in the vessel may pass freely through the capillary.

After about ten minutes temperature equilibrium is obtained.

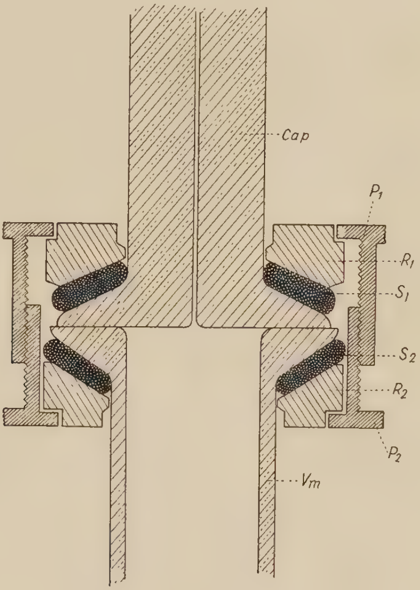

Fig. 5. Longitudinal section through the uniting mechanism. For explanation see Fig. 4.

Next the dilated part at the upper end of the capillary is filled with the index fluid[2] and the unit is connected to the measuring system by means of the standard joint J and a rubber tube.

In order to get the index fluid into the capillary lumen, suction is applied by means of the syringe R_s (Fig. 2). A few air bubbles are sucked through the index fluid and while atmospheric pressure is being reestablished, the index fluid rushes into the capillary. One easily finds out how much air should be sucked out to make the index meniscus travel the right distance down the capillary. In our experience this method of adjusting the index meniscus is much more convenient than the painstaking adjustment of an index *drop* in the capillary.

During this part of the procedure the manometer system is not used, the stopcock K_5 (Fig. 2) being in position 2, whilst K_6 is open.

By means of the vertical movements of the clamps the index menisci are—under atmospheric pressure—successively brought into view of the microscope and the clamps are locked in this position.

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[1] A mixture of vaseline, paraffin wax (melting point 50° C.), non-vulcanized rubber and resin is used by us. This grease is liable to oxidation and may advantageously be substituted by silicone grease.

[2] The index fluid is a watery solution of 0.9 per cent NaCl with 0.1 per cent T-pol.

Next K_5 is put in position *3* and K_6 is closed. K_4 is brought into position *b*; K_3 is closed—this puts the vertical manometer out of action; K_1 and K_2 are opened.

Now the whole system is independent of changes in atmospheric pressure.

By opening the stopcocks k_1, k_2, etc., each of the units can be connected to the manometer system and measurements can be made.

For metabolism measurements the inclined manometer is used. The use of the vertical manometer will be shown below. Pressure in the system can be controlled by the screws *A* and *B* for coarse and fine adjustment respectively (10).

Measurement is made by successively adjusting each index meniscus accurately on the horizontal hair in the eyepiece of the microscope by means of the screws *A* and *B* after the corresponding stopcock *k* has been opened, and by reading the pressure on the inclined manometer. Adequate wetting of the capillary wall should be ensured by moving the index fluid meniscus up and down before each reading.

For the calculation of the amount of O_2 consumed, the gas volume of the measuring vessel should be known. This volume is measured by changing the unknown vessel volume by a known quantity, without changing the amount of gas in it. From the pressure difference induced by this change the gas volume of the vessel can be calculated.

This measurement is made in the following way:

The capillary of the unit is calibrated. The index meniscus in it is moved over a known volume and the pressure difference caused by this displacement is measured. As relatively large pressure changes are involved, the vertical manometer is used; in this case its right limb is open to the atmosphere (K_4 in position *a*; K_3 open; K_1 and K_2 closed).

N.B. The pressure difference read on the manometer should be corrected for the lengthening, resp. shortening, of the fluid column in the capillary when the index meniscus is lowered, resp. raised.

Calculations. For calculation of the amount of O_2 used during the experiment Warburg's equation for constant-volume respirometry has been adapted to our purposes:

$$x = h \frac{a P_1}{(p' + 0.946\, k s_t) P_0} \frac{273}{T} \cdot$$ For derivation of this equation see Prop (15).

$$\frac{a P_1}{(p' + 0.946\, k s_t) P_0} \frac{273}{T}$$ being the *vessel constant.*

x = quantity of O_2 consumed between two measurements at time 1 and time 2, corrected to 0° C and 76 cm Hg.

P_1 = pressure in measuring vessel read at time 1, in cm Brodie.

P_2 = ,, ,, ,, ,, ,, ,, ,, 2, ,, ,, ,,

$h = P_1 - P_2$

a = volume by which the unknown constant volume is changed for determination of the gas volume of the vessel; this determination is included in the equation given above.

p' = pressure difference measured on the vertical manometer if the constant volume is changed by a volume a, in cm Brodie.

$0.946\ k s_t$ = pressure difference caused by the change in length of the fluid column in the capillary if the constant volume is changed by a volume a. In this, k is the length difference of the fluid column, s_t is the specific gravity of the index fluid, whilst factor 0.946 corrects this product to a value expressed in pressure of cm Brodie.

T = working temperature in degrees Kelvin.

P_0 = pressure of 76 cm Hg in cm Brodie.

It should be noted that the correction factor for the quantity of fluid in the vessel, present in the Warburg equation, is missing in the one given here. The reason for this is that the gas dissolved in the fluid in the vessel occupies a *virtual* space in which it obeys Boyle's law. This quantity of gas is included in our measurement.

Sensitivity and stability of the volumetric part. Using a vessel volume of 150 mm³ and a reading accuracy of 1 mm on the inclined manometer (1 : 10), corresponding with a pressure of 0.1 mm Brodie, a sensitivity of the order of $1 \cdot 10^{-3}$ mm³ is obtained.

As respiration measurements are extended over some space of time, the stability of the method should be taken into account. Even if the vessels are empty, or still better, only provided with a wet filterpaper strip to ensure the right vapour pressure, there is always a slight decrease in gas volume, so much more as the working temperature is higher. No reason for this effect could be found, but many workers on microrespirometers report

TABLE I

Working temperature 38°C.

Measuring vessel	Deviation from 0-line per hour	Mean deviation per hour	Deviations from mean	Standard dev. from mean per hour
1	$1.2 \cdot 10^{-2}$ mm³	$1.2 \cdot 10^{-2}$ mm³	0.0	$0.5 \cdot 10^{-2}$ mm³
2	0.5		0.7	
3	1.8		0.6	
4	1.3		0.1	
5	1.0		0.2	
6	0.7		0.5	
7	1.0		0.2	
8	1.5		0.3	
9	1.3		0.1	
10	2.0		0.8	

similar experiences. Oxidation of the grease used for tightening the joint may be one cause; in that case it might be overcome by using silicone grease.

The decrease in gas volume reported always takes place at nearly the same rate. In order to eliminate this interfering factor as much as possible, a mean rate of decrease and the standard error of this mean have been calculated from a number of blank measurements. The accuracy of measurement can be enhanced by taking these values into account.

In Table I the data are given for *our* apparatus; they may, however, not be valid for another apparatus, even constructed in a similar way. Accordingly, the blank measurements should be repeated for any new apparatus.

Measurement of CO_2-production

If CO_2 is absorbed by a $Ba(OH)_2$-solution, the electric conductivity of the latter decreases, as $BaCO_3$ is precipitated, thus causing a reduction in the number of ions. As this decrease in conductivity is a function of the quantity of CO_2 absorbed per quantity of solution, a method of measuring the amount of CO_2 involved in some chemical or biological process may be based upon it.

It is astonishing that since the time Fenn (5) and von Ledebur (12) introduced this elegant method into microrespirometry it has attracted so little attention.

If R_m is the electric resistance of a fluid contained in a vessel, the specific conductivity $1/R_s = 1/p\,R_m$, p being a constant, the *vessel constant*, depending on the geometry of the vessel and the electrodes. The change in $1/R_s$ of a given volume of $Ba(OH)_2$-solution is a measure of the quantity of CO_2 produced by the respirating object and absorbed by the solution.

The fewer $Ba(OH)_2$ molecules present, the higher the *percentage* that will be removed by the same quantity of CO_2. Measurement sensitivity will consequently reach its maximum when a minimal number of $Ba(OH)_2$ molecules are present.

This can be achieved in two different ways:

1. By using a very dilute solution,
2. By using a minimum amount of solution of moderate concentration.

N.B. One should not go too far in diminishing the amount of $Ba(OH)_2$, because there must be enough to absorb *all* CO_2 released during the whole experiment.

If very dilute solutions are employed, as Fenn and von Ledebur did, the following difficulties are encountered:

a. In the relatively large quantity of fluid used, the necessary uniform mixing should be ensured by stirring.

b. Even if no CO_2 is present, conductivity shows a considerable drift, for which no cause could be found.

In our experience this drift is considerably less if a stronger concentration (at least ten times the concentration used by the above mentioned authors) of $Ba(OH)_2$-solution is used.

In consequence the amount of solution used must be reduced. Therefore, in measurement vessels of 150 mm³, only 12 mm³ of a n/5–n/10 $Ba(OH)_2$-solution is used. In this small quantity no stirring is required, diffusion alone ensuring uniform mixing all through the solution.

The $Ba(OH)_2$-solution should present a large surface to the gas in the measuring vessel in order to obtain a maximum rate of CO_2-absorption. On the other hand, the fluid volume should always have the same geometry with respect to the electrodes, as otherwise the vessel constant is not reproducible. These conflicting demands have been met with in the following manner:

1. If the vessel diameter does not exceed 3 mm, the free surface of the solution always assumes the same form under the influence of capillary force; thus uniform geometry is ensured.
2. The electrodes should be close to the bottom of the vessel, i.e. as far as possible from the free surface of the fluid. As the greater part of the electric current passes far from this surface, influence by chance deformations of this surface is reduced.
3. Each vessel diameter requires its own amount of solution. If the amount of fluid is too small, p is hardly reproducible, whereas in case there is too much of it, the diffusion path from the free surface to the bottom is unduly increased, which impairs accurate measurement. In vessels with a diameter of 3 mm, a fluid volume of 12 mm^3 was found to be the best compromise. Using this volume, no greater deviation from the main vessel-p than 3 per cent was found.

The electrodes are loops of 0.2 mm platinum wire, 3 mm long, projecting from the glass bottom (Figs. 7 and 11). They are covered with platinum-black (11).

The vessel constant p is determined by filling the vessel with 12 mm^3 of a standard solution of KCl of known specific conductivity.

Measurement is effected in a Wheatstone-bridge circuit with alternating current 1000 c/s. A variable compensating condenser C (Fig. 6) enables a well defined reading minimum to be obtained. As a minimum indicator a cathode-ray oscillograph (Philips G.M. 3152) is used, which offers the advantage of a very precise reading of the bridge minimum.

Calibration of CO_2-measurement by means of "slow diffusion". In our microrespirometer, decreases in gas volume and in electric conductivity can be measured simultaneously. This offers a means of calibrating the CO_2-measurement.

A tube filled with pure CO_2 and sealed at its end by a drop of index fluid, is brought into the measuring vessel. It is fixed in the vessel by means of a stainless steel spring. The index fluid can move freely in the tube and CO_2 diffuses slowly through it to the space of the measuring vessel, where it is absorbed by the $Ba(OH)_2$ (Fig. 7). The decrease in CO_2-volume is measured by the volumetric system; as the fluid in the CO_2-tube can move freely up and down the tube, the gas volume *in* this tube is measured together with that in the vessel. The decrease in electric conductivity corresponding with the volumetrically measured decrease in CO_2-volume is determined on the bridge. In this way a calibration curve is constructed from the parallel measurements of several vessels (Fig. 8). Note the good agreement in the different vessels.

N.B. Such a calibration curve applies to one special working temperature only. For every other temperature another special calibration should be made.

When the CO_2 in the tube becomes exhausted, the corresponding part of the calibration curve shows a curved tail. This tail is caused by the last CO_2 dissolved in the liquid seal of the tube and extracted from it by the $Ba(OH)_2$. This part of the curve, which can be clearly distinguished, should be ignored.

The $Ba(OH)_2$-solution is carefully brought into the vessel by means of a micropipette provided with a long fine tip, which is kept accurately centered in the vessel to prevent "creeping up" of the fluid between this tip and the vessel wall (10). The pipette tip should not touch the electrodes, as any displacement of the latter will alter the vessel-constant p.

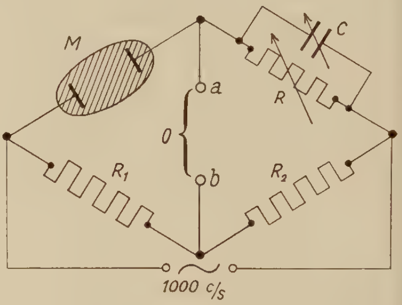

Fig. 6. Wheatstone bridge circuit for measurement of fluid conductivity. M = measuring vessel. O = cathode-ray oscillograph used as a zero indicator. R_1 has the same value as R_2: the bridge is symmetric. R = decade resistance-box 10,000 — 1.0 ohms. C = condenser continuously variable from 40,000 — 40 pF.

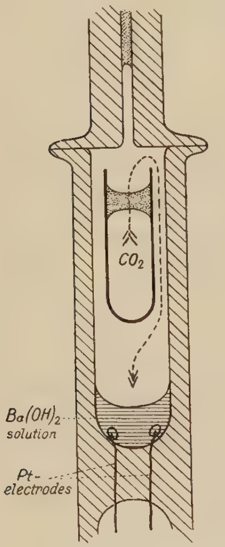

Fig. 7.

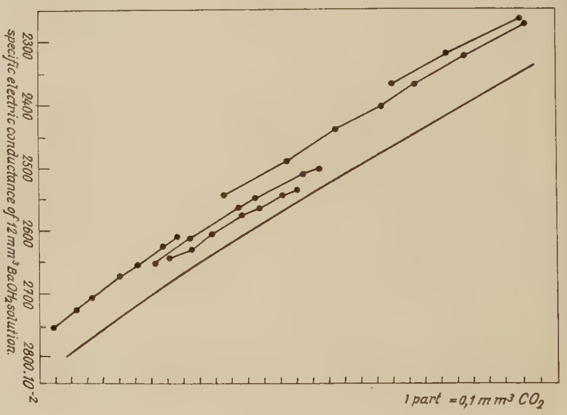

Fig. 8.

Fig. 8. Calibrating curves indicating how in 5 vessels the specific conductivity of 12 mm³ of $Ba(OH)_2$-solution decreases when CO_2 is absorbed, determined in the way given in Fig. 7. The dots connected by lines are the values measured in each vessel. Working temperature 38.0 °C. N.B. Only the *slope* of the lines is important; their height was chosen arbitrarily.

Sensitivity and stability of CO_2-measurement. The sensitivity of the method depends on the quantity and concentration of the $Ba(OH)_2$-solution. A quantity of 12 mm³ n/5–n/10 was found suitable for 150 mm³ vessels (3 mm diameter). Using this amount, a sensitivity of 0.01 mm³ is obtained if resistance is read with an accuracy of 0.1 Ohm.

In the same way as for the O_2-measurement, the mean drift of the zero point and the standard deviation from this mean, expressed in mm³ CO_2, have been determined.

TABLE II

Working temperature 38°C.

Measuring vessel	Deviation from 0-line per hour	Mean deviation per hour	Deviations from mean	Standard dev. from mean per hour
1	$1.4 \cdot 10^{-2}$ mm^3	$1.6 \cdot 10^{-2}$ mm^3	0.2	$0.7 \cdot 10^{-2}$ mm^3
2	0.6		1.0	
3	1.4		0.2	
4	1.2		0.4	
5	1.0		0.6	
6	0.8		0.8	
7	1.7		0.1	
8	1.2		0.4	
9	1.2		0.4	
10	1.5		0.1	
11	2.0		0.4	
12	3.4		1.8	
13	2.5		0.9	
14	2.0		0.4	

The water bath. For both volumetric and electric measurements a constant temperature is essential. Therefore, measurements are performed in a water bath which is kept at a constant temperature. To ensure a good consistency of this temperature the following measures are taken:

1. A sensitive contact-thermometer or a toluene-regulator is used for temperature control.
2. A heater of minimal heat-capacity is used (a 40 Watt electric bulb, according to Holter (10).
3. The water in the bath is stirred vigorously.
4. The ambient temperature should not differ more than 2° C from the bath temperature (constant-temperature room).
5. The water surface exposed to the atmosphere is reduced to a minimum in order to diminish cooling by evaporation of the water. This evaporation is greatly reduced by covering the bath with a closely fitting lid, provided with a narrow slit which admits the row of measuring vessels. As the rim of this slit dips into the water, only a very limited area of the water surface is in contact with the atmosphere (Fig. 9).

By these measures the temperature is kept constant within 0.002° C for a period of at least 12 hours when using a toluene-regulator (and within 0.01 °C when using a contact thermometer). Within the bath no local temperature differences could be detected.

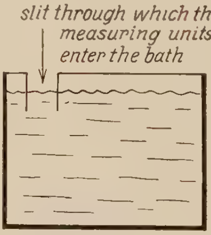

Fig. 9.

Application of the apparatus for the determination of the Respiratory Quotient (R.Q.) of fibroblasts cultured in vitro

Though many authors measured O_2-respiration, and aerobic and anaerobic glycolysis of tissue cultures (17, 16, 9, 7, 18, 3) R.Q. measurements—valuable information about *which* substrate from the medium is metabolized—were not made. This lack may be attributed to technical difficulties: it is far from easy to distinguish between *respiratory* CO_2 and "*glycolytic*" CO_2 liberated from the medium bicarbonate by lactic acid produced by glycolysis.

Two methods are available:

1. measurement in a carbonate-free milieu,
2. measurement of the increase of carbonate in the culture vessel.

The first method was applied.

EXPERIMENTS WITH CHICK HEART FIBROBLASTS

Materials and methods. Chick heart fibroblasts were cultured after the usual "hanging drop" procedure in a medium consisting of equal parts of chick plasma and extract of 9-day old chick embryos (E.E.).

The cultures used for measurements were not younger than the sixth passage.

Twenty-four hours before measurement the cultures were cut out of the plasma clot and placed on perforated mica strips (3 mm wide; 15 mm long; 3–4 cultures per strip) and fixed to these with a minimal amount of plasma and E.E.

After clotting, these strips were submerged in a medium consisting of horse serum, E.E. and Paneth-Compton-solution[1] (1:1:2). This medium is contained in the cavity of a tissue-culture depression-slide which is placed in a Petri dish, bottom and lid of which are covered with filter paper soaked with a 0.6 per cent NaOH-solution.

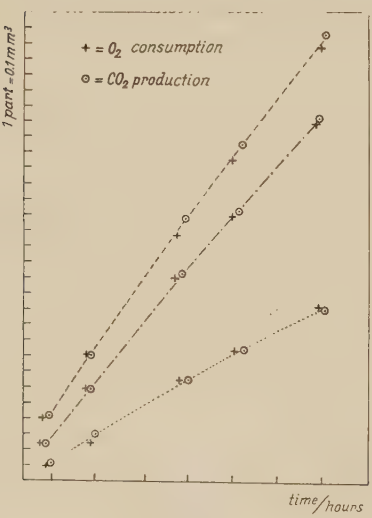

Fig. 10.

In this Petri dish—cemented air-tight by paraffin wax—the cultures remain 24 hours at 38° C, during which time the medium and cultures are liberated from carbonate without undue change in pH.

After this period the strips are transferred to the measuring vessels and R.Q. is determined over a period of 6 hours in the way described. The thin plasma clot contains enough foodstuffs for the cultures during the period of measurement.

Results

From Fig. 10 it can be seen that the R.Q. of heart fibroblasts treated in the way described is very near 1.0. The experiment was carried out with cultures of the 24th passage.

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[1] Paneth-Compton-solution is a phosphate-buffered physiological saline (6).

DISCUSSION

In an ideal metabolism measurement the tissue under investigation should conform to the following requirements (15):

uniformity in metabolism,
consistency in metabolism,
"physiological state".

It will be considered how far in the experiment described the tissue approximates these requirements.

Uniformity in metabolism is aimed at by the use of pure strains of fibroblasts. In the case of proliferating cultures, however, we deal with cells in mitosis and cells in the intermitotic phase. So at least two different types of metabolism must be expected. But as it is likely that during mitosis, glycolysis is the main source of energy for the cell (12, 1, 13), the metabolism of these cells may be neglected in respiration measurements, for no O_2 is taken up and no CO_2 is produced. The R.Q. measured should be attributed to the intermitotic cells.

Consistency in metabolism is ensured by consistency in environmental conditions.During twenty four hours before measurement the cells are kept in circumstances identical with those during measurement. During this time the cultures get adapted to CO_2-deprivation, the damage caused by the transfer to the mica strips is overcome, and maximum growth activity is obtained; this activity is known to be constant after this time for at least 24 hours (6).

From the curves of Fig. 10 it can be seen that indeed during the period of measurement no change in the metabolism does occur, the curves being approximately straight lines.

"Physiological state." If one should require that cells in the experiment should be in a state fully comparable with any state they are in in the organism, experimenting had better be stopped. Nevertheless, we must try to approximate this state as we are primarily interested in the physiological life process of the cells *in vivo*, though for the sake of experimental possibilities we are compelled to study it *in vitro*. The first and chief requirement that should be fulfilled is, that there should be the *possibility of continuation of the life process for a normal span of time* (15).

In the experiments described above this requirement was fulfilled: the cultures proved to be completely viable after the experiment when transferred to a normal hanging drop.

CO_2-absorption, and combined with it the relatively high level of phosphates in the medium, are the chief deviations from ''normal'' conditions for the cultures. CO_2 is known to be not only a final product of metabolism, but it may be used by some cell types in the synthesis of other metabolites (4, 14). Therefore after the experiment the cells were checked for degeneration signs: a slight increase in fat vacuoles and neutral red vacuoles was found, scarcely more than in parallel controls without CO_2-absorption; mitochondria—important links in respiration—did not show any change when stained vitally with Janus black.

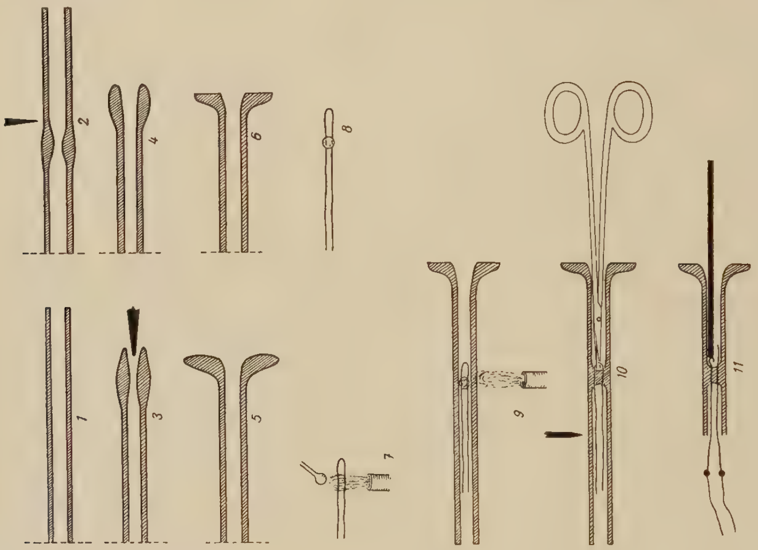

Fig. 11. Mode of construction of the measuring vessels. 1. Glass tube 3 mm diameter. 2. The middle part of the tube is shrunk to thicken the walls and it is cut off at the point indicated. 3, 4, 5. The shrunk part is made into a flange. This may be done by free hand or in a lathe. Subsequent thorough cooling in asbestos powder is essential. 6. The flange joint is ground flat with emery of increasing fineness and subsequently polished with polishing red. 7, 8. A hairpin of 0.2 mm platinum wire is provided with a glass bead. 9. The beaded hairpin is brought into the tube (6) and melted into it. 10. By means of a fine pair of scissors the hairpin bend is cut and the glass tube is cut off at the point indicated. 11. The ends of the platinum wires are curled into loops. Leads are soldered to the other ends, and a flexible tube protects these leads. Insulation tube prevents the platinum wires from touching and thus causing short circuit.

SUMMARY

I. A microrespirometer was described with the following characteristics:

1. O_2-consumption and CO_2-production may be measured simultaneously and independent of each other. O_2-consumption is measured volumet-

rically. CO_2-production is measured by determining the change in electric conductivity of a bariumhydroxide solution.

2. Measurement sensitivity is 0.001 mm^3 for O_2, and 0.01 mm^3 for CO_2.

II. The Respiratory Quotient of proliferating fibroblasts *in vitro* was measured and it appeared to be 1.0.

The conditions prevailing in these kinds of experiments are shortly discussed.

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TESTING THE PERFORMANCE OF A SIMPLE FREEZE-DRYING APPARATUS FOR HISTOCHEMICAL PURPOSES

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Although there is abundant literature with regard to freeze-drying equipment for histological purposes, relatively little has been published about the manner in which the performance of the various devices has been tested.

Yet, it is of importance to know with certainty when the frozen tissue can safely be considered dry in order to obviate either unduly prolonged drying periods or the examination of incompletely dried objects.

Thus, even the simplest apparatus for histological freeze-drying should provide some means of following the drying process.

In connection with the testing of the performance of a freeze-drying apparatus of a simplified design we have investigated the following variables: a. weight of the frozen block during drying, b. temperature of the tissue block, and c. variation of pressure in the system with pumps temporarily off.

The present paper first deals with the design of the apparatus; next, the findings on the above mentioned variables will be presented; finally, the influence of residual air pressure on the drying rate will be discussed.

I. The freeze-drying apparatus (see Fig. 1) essentially consists of a U-shaped pyrex-glass tube, one leg of which bears a stopcock and is connected to the vacuum pump (in our case either a two-stage rotary pump or a small oil-diffusion pump with a rotary fore-pump). Part of the other leg, which is the drying tube proper, is surrounded by a copper mantle, which, in turn, is enclosed in a sealed off vacuum jacket. Another piece of wide-bore copper tubing envelopes this jacket from about the middle to well below its lower end.

The upper end of the drying tube is provided with a flange joint, to which the gauge head of a Pirani vacuum meter is sealed with vacuum grease.

The lower part of the U-tube is cooled with a dry ice-acetone mixture contained in a wide-mouthed vacuum flask. (The two projecting legs of the tube are made to pierce a cover of sheet rubber (the dentists' rubber dam), which excludes atmospheric moisture from the cold parts.) The submerged parts of the tube are thus cooled down to the temperature of the cooling mixture (*viz.* −70° C), but the temperature of the drying tube, which is isolated from the dry ice by the vacuum jacket, will be between −70° C and room temperature. The inner copper tube, by virtue of its heat conducting properties, equalizes this intermediate temperature over a fairly long distance along the drying tube, whilst the outer mantel of the same material serves to diminish the effect of the gradual lowering of the dry ice-acetone level caused by the evaporation of carbondioxide. By adjusting the relative heights of the copper tubes a temperature of −40° to −45° is easily obtained within the drying tube. Once set, the temperature has been found to remain within this range in spite of changes in room temperature and the pressure in the drying tube, even if fresh CO_2 is added only once in 24 hours.

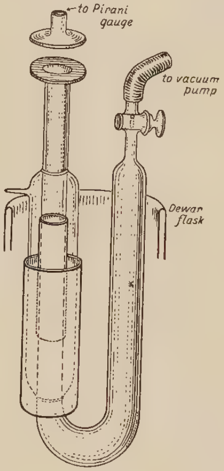

Fig. 1. Diagram of the freeze-drying apparatus.

The rapidly frozen objects are introduced into the apparatus after removal of the Pirani gauge head. To this end they are contained in wire-gauze baskets suspended by cotton threads which can be hooked to the upper end of the drying tube (not illustrated).

Next, the Pirani gauge is sealed to the flange joint and the pump turned on. Pumping is discontinued only during the night and the material is considered dry if during this period the pressure in the system does not rise appreciably. The reasons for this will be set forth in the following section. (Actual drying times at drying-tube temperature between −40° and −45°: a. pieces of rat liver about 2 mm thick: 36 hrs; b. rat lung lobes (inflated): 2½ days; c. young salamander heads, cut off just behind the eyes, about 2 × 3 × 4 mm: 4½ days).

If the dry material is to be embedded in paraffin wax it is rapidly transferred to a *separate* vacuum-embedding type. It should be noted, that a comprehensive study of the histological appearances of the biological specimens after different modes of drying has not yet been made. As judged from routinely stained slides, however, the above mentioned procedure yielded quite satisfactory results in those parts of the objects that had been frozen rapidly.

II. For the purpose of testing the performance of the apparatus described in the foregoing section the following additions have been made: first, a vacuum-tight torsion balance has been fitted to the flange of the drying tube (see Fig. 2) and second, the object carrier has been provided with a thermojunction.

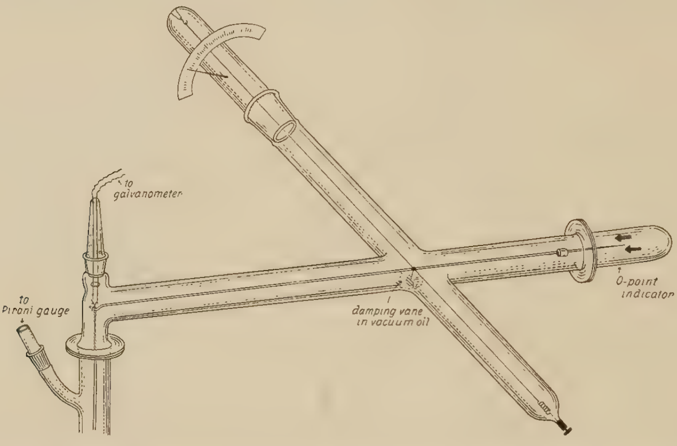

Fig. 2. Vacuum-tight torsion-balance to be attached to the apparatus depicted in Figure 1.

The construction of the simple steel wire torsion balance needs no comment. It has been found to respond linearly in the range employed and its readings were reproducible within a few per cent of the lowest values measured.

The object carrier consists of a small piece (2 × 4 mm) of 0.2 mm thick copper sheet onto which the ends of a 0.1 mm copper and 0.02 mm constantan wire have been soldered. With their upper ends these wires, which act as suspending wires for the object carrier, are attached to a hook which fits the beam of the balance.

Platinum wires, connected to the ends of the constantan and copper wires respectively, project in opposite directions from the upper end of the hook. Two similar wires extending from leads sealed through the male part of a cone joint can be made to engage those on the hook by merely turning the joint.

Thus both the weight of the object and its temperature can be ascertained without disturbance of the vacuum.[1]

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[1] The temperature of the upper ends of the copper and constantan wires has been assumed to be equal to room temperature. Calibration of the entire measuring circuit revealed an accuracy to 1° C at −40° C.

After the quickly frozen object has been introduced into the drying tube and the pump set to work the course of events is generally as follows (see Fig. 3): The weight of the object will decrease, at first at a rapid rate and later on more slowly, until it reaches a fixed level. After a sharp initial drop, the temperature of the block will rise to a level still well below the temperature of the drying tube. It is only after the weight curve has levelled out that the temperature will rise rather quickly to that of the drying tube.

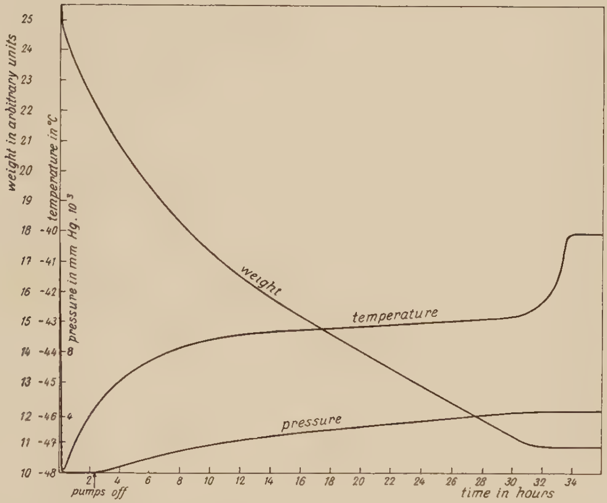

Fig. 3. Graphic presentation of the findings when weight and temperature of the object, and pressure in the system are being measured during freeze-drying.

If the pump is kept running the pressure in the system will remain nearly constant, but as soon as the stopcock of the apparatus is closed the Pirani gauge will indicate a slow rise of the pressure. If no appreciable leakage takes place the pressure curve, too, will level out when drying is completed.

The shape of the weight-time curve depends on the nature of the object. It is a steep and nearly straight line when pure ice is being evaporated, whereas in the case of biological objects its slope will decrease rapidly during the first hour or so of the drying process. Curves similar to those of biological objects can be obtained by the evaporation of pure ice from tight pads of filter paper.

The temperature of the object (or rather of the object carrier) is governed by the influx of radiant heat from the drying-tube walls (and to a probably very small extent through conduction along the thin suspending wires) and by the loss through radiation and withdrawal of latent heat of evaporation.

After the object has been introduced into the drying tube at normal air pressure it assumes the drying tube's temperature as no appreciable evaporation takes place.

As soon as the vacuum begins to develop, however, the superficial layer of ice will evaporate quickly, withdrawing much heat, hence the initial drop in temperature.

The further course of the temperature curve reflects the much slower rate of evaporation of ice from within the object.

The gradual rise in pressure during the drying process with pumps off is to be ascribed to the release of gases from the object. Most probably, these gases are trapped in the object during the initial quick freezing. Apparently they are released only according as the ice evaporates, for no sooner has the object attained constant weight than the rise of pressure in the system stops.

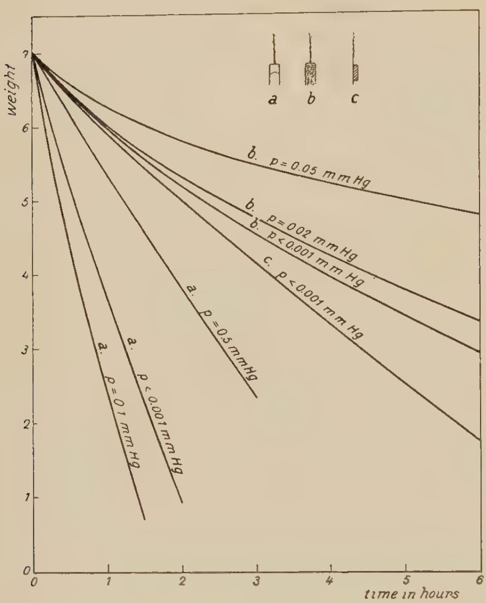

Fig. 4. Drying rate of different objects (a: pure water, b: pure water absorbed by filter paper, and c: rat liver) at various residual air pressures.

The recorded correlation between loss of weight, temperature of the object and release of gases having been confirmed by many experiments we feel justified in selecting one of these variables for the purpose of establishing the end point of the drying process in an apparatus for routine use. Measurement of the amount of gas evolved with pumps turned off is by far the most practical choice.

III. With the apparatus described we have investigated the influence of residual air upon the drying rate of a. droplets of pure water, b. pure water adsorbed by filter paper and c. rat liver.

In the case of a. the object carrier had been modified to form a short, thin-walled cylindrical vessel. Ten cubic millimetres of water were carefully deposited on the bottom of the vessel and, after freezing, the object carrier, its open end pointing downward, was introduced into the drying tube. For the experiments mentioned under b. the same object carrier was used, tightly stuffed with filter paper. Small (2 × 3 × 4 mm) pieces of rat liver were investigated while attached to a plane object carrier (see inset in Fig. 4, a, b and c).

The drying rates of pure ice at three different air pressures are plotted in Fig. 4, a. In all cases the temperature of the drying tube was maintained at about −40° C. Yet, the object carrier attained temperatures of —55° C with the best vacuum (< 0.001 mm Hg). At the optimal pressure (0.1 mm Hg) the temperature of the object fell to −50° C, whereas at 0.5 mm Hg it did not get below −45° C.

Water, frozen after being adsorbed by filter paper, behaves quite differently. It evaporates far more slowly (accordingly, the temperature of the object carrier does not differ more than 4° from that of the drying tube) and moreover the drying rate is greatest at the lowest air pressure, though in our experience not much is gained by lowering the pressure below about 0.003 mm Hg.

Biological specimens dry at about the same rate as the object mentioned under b. (see Fig. 4, c).

DISCUSSION

The apparatus is believed to offer some advantages over existing designs: a. One coolant is employed, both for trapping the water and for cooling the object. Stowell's (10) apparatus shows the same feature but it needs liquid nitrogen, which is not so easy to obtain, keep and handle as carbondioxide. Yet, the use of dry ice for cooling the condenser is justified, the vapour pressure of ice at −78° C being less than 0.001 mm Hg, as compared with 0.09 mm Hg at −40° C. Thus, the difference between the vapour pressures in the evaporator and the condenser can be but little affected by lowering the temperature of the latter. Indeed, on abandoning cooling by liquid air we found no appreciable loss in the drying speed of biological objects.

b. No separate water trap is needed either for drying the atmospheric air upon relieving the vacuum or for protecting the mechanical pump.

c. The progress of the drying process can be followed by reading the Pirani gauge. Though this method had in some form been applied earlier (3, 6, 11) its validity had yet to be proved by comparison with other methods. This seems to have been made possible for the first time through the addition of weighing and thermometry to the measurement of the pressure, though an apparatus allowing the same variables to be measured is described by

Ede (2). His results, however, are not comparable to ours as he was interested in the drying of foodstuffs and worked on a much larger scale and at higher temperatures of the object.

The long duration of the process constitutes a serious drawback of freeze-drying as a histological technique.

Some new devices have recently been claimed to perform the drying in a fraction of the time commonly considered necessary. Glick and Malmstrom (5), for instance, find drying times of no more than two hours at tissue temperatures of -40° C. It appears, however, that their criterion for dryness is less unequivocal than ours. The excellent histological preservation of the tissues, on the other hand, seems to invalidate any objections raised against their technique.

One might try to cut down the time needed for freeze-drying by improvement of the apparatus along the lines dictated by the physical principles involved (7), but there soon comes an end to this, as the major impediment to quick drying lies in the object itself (4). In the first place it is difficult to prevent the drying object from cooling too much below the desired temperature through withdrawal of latent heat of evaporation. Heat can be supplied to the object through either conduction or radiation. In the former case the object should be thin as it is a poor thermal conductor and broadly attached to a support kept at the required temperature. Quite often the nature of the object prohibits this measure, apart from the fact that placing a slice of tissue upon a flat support diminishes its evaporating surface by half and doubles the ultimate thickness of the dry shell.

The object will receive the maximal amount of heat through *radiation* if it is surrounded as completely as possible by the radiating shield of the temperature selected. In our apparatus this has been achieved by suspending the object halfway a relatively long tube of uniform temperature. It seems that some freeze-drying apparatuses of recent design (e.g. (10, 8, 1)) are less favourable in this respect, as in these cases the objects are largely exposed to low temperature surfaces.

One might reasonably try to supply heat to the object by filling the drying tube with a heat conducting gas at an appropriate pressure. (Hl. de Vries, personal communication from a more complete theoretical and experimental study of the physical aspects of the freeze-drying of histological objects.) We have investigated this possibility in our apparatus in the case of air and found the expedient to work well if pure ice is being dried. Within the range of the Pirani gauge (0.5–0.001 mm Hg) no optimal air pressure could, however, be established for the drying of objects in which the diffusion of water vapour is impeded by an ever thickening shell of dried matter (compare Fig. 4, curves a with b (and c)).

Thus, in case a (pure water), the resistance to diffusion of water vapour offered by air at 0.1 mm Hg as compared with lower pressures is more than balanced by the better heat conductance of the air at the former pressure. This does not hold for still higher air pressures, as is evidenced by the 0.5 mm Hg-curve. The discussion of the behaviour of the objects b and c at different air pressures should take into account the second limiting factor inherent to biological objects: the development of a dry shell. Through its presence the drying process involves two stages:

a. conveyance of water vapour from the drying interface to the surface of the dry shell, and

b. diffusion of the vapour from the surface of the dry shell to the condenser surface, this stage being the only one if pure ice evaporates.

A comparison of the slopes of the a- and b-curves respectively (Fig. 4) clearly shows the magnitude of the influence of the first stage, *viz.* the hindrance to the water-vapour diffusion offered by the dry shell. In fact, the drying rate of the filter paper object soon becomes $^1/_4$ to $^1/_{10}$ of that of the pure-ice object, in spite of the much lower temperature attained by the latter. The differences in drying rates of the filter paper object at various residual air pressures are evidently too great to be attributed solely to variations in the resistance to water-vapour diffusion in the drying tube (stage b). Consequently it must be assumed that the air molecules present in the pores of the dry shell interfere in some way or other with the transport of the water molecules (stage a), though the free mean path of the water molecules in air at the pressures used is greater than the entire thickness of the dry shell.

Gersh (4) presents a formula in which the drying rate is inversely proportional to the square of the shell thickness. Yet, in our experience the slope of the weight-time curve of biological tissues does not suggest such a relation between shell thickness and drying rate. In fact, after a fairly rapid decrease the slope may for some time be nearly constant (Figure 3 and Figure 4, c). It seems as if the major obstacle to diffusion of water vapour is located in the outer shell of the tissue block, the deeper layers of dry tissue only contributing insignificantly to the total resistance. This supposition is in keeping with the well-known fact that microscopically visible ice-cristal lacunae are smallest in the most rapidly frozen superficial layer of the objects (9). If the importance of the pore size, as influenced by the freezing velocity, be confirmed, one should reckon with a dependence of the drying rate on the quick-freezing technique employed.

CONCLUSIONS

1. Freeze-drying of histological objects can be performed in a simple apparatus, which needs a relatively inexpensive pump and carbondioxide as a refrigerant.

2. As the major obstacle to the attainment of shorter dehydration-times is in the object itself, complicated and expensive elaboration of the equipment is not justified.

3. The termination of the process should be well-marked; reading a manometer with pumps turned off is a practicable method.

SUMMARY

A simple and inexpensive apparatus for freeze-drying histological specimens is described. Its performance has been tested by measuring three variables: weight and temperature of the block during drying, and rise of pressure in the system with pumps off. The latter measurement has been found to be a practicable and reliable means of determining the end of the drying process. The dependence of drying rate on pressure in the system has been investigated in the cases of pure ice and artificial and biological objects.

In this investigation, the histological appearance of the tissues, though good after the procedure finally adopted, has not been used as a criterion of dryness.

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THE EFFECT OF SOME OXIDIZING AGENTS UPON FERTILIZATION AND ENSUING DEVELOPMENT OF THE SEA URCHIN EGG

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Dilute solutions of sodium periodate in sea water have a striking effect on sea urchin eggs which are refractory to fertilization. Before fertilization such eggs are often anisodiametric. In contact with a periodate solution in sea water they assume almost instantaneously a spherical form. Observations in the centrifuge microscope have also revealed that the rigidity of the egg surface decreases considerably subsequent to exposure to periodate (7).

If eggs are inseminated in presence of e.g. $2–4 \cdot 10^{-5}$ M sodium periodate in sea water they undergo in general a perfect fertilization, even if the untreated eggs show a low percentage of fertilization with incomplete membrane formation and strong contraction of the cytoplasm (8, 16).

Even a pretreatment with this reagent suffices to insure normal fertilization. The treatment with periodate is also reflected in the ensuing cleavage and development to later stages.

The refractoriness in question is certainly often due to an underripeness of the eggs as could be shown particularly for *Psammechinus miliaris* from Kristineberg, the Zoological Station on the Swedish west coast (15). But also in work at the Stazione Zoologica, Naples, the sea urchin eggs showed refractoriness during certain breeding seasons despite the precautions taken e.g. by inseminating and raising the eggs in sea water brought in glass baloons from the open sea. During such seasons the treatment with periodate constituted a useful method of getting normal fertilization and uniform development to the pluteus stage. Only very rarely eggs (of *Arbacia lixula*) were insensitive to the improving action exerted by periodate.

Certain substances like heparin, dissolved substance of the jelly coat of sea urchin eggs, certain fractions of extract from *Fucus vesiculosus* etc. were found to act as inhibitors of the fertilization of the sea urchin. The inhibitory effect of these substances could be removed by means of periodate (5, 21).

Periodate is an oxidizing agent; particularly its oxidation of carbohydrates has constituted a valuable tool in the study of these substances, see (14). It seemed therefore of interest to inquire whether periodate could be replaced in its action on fertilization and development by other oxidizing agents. Experiments were carried out on the action of iodate, iodine, hydrogen peroxide, potassium ferricyanide and iodosobenzoate. Parallel observations on the effect of periodate were also made. Not only the influence of the agents on the fertilization, but to some extent also their effect on the ensuing development will be considered in this report. For some previous results with iodate, see (8).

For the experiments *Paracentrotus lividus* and *Arbacia lixula* were used. The eggs of *Arbacia lixula* exhibit in general a closely adhering fertilization membrane. Such eggs are very suitable for demonstrating the action of membrane improving substances. In *Arbacia lixula* the eggs exhibit either a closely adhering or a well elevated membrane, whereas intermediate stages are rare (19). In *Paracentrotus lividus*, on the other hand, intermediate steps are more common, a circumstance that renders the classification somewhat more difficult.

METHODS

The preparations of the egg and sperm suspensions were made according to current practice, see e.g. (5).

The eggs were either pretreated before fertilization in presence of the oxidizing reagents or the fertilization occurred in the presence of these reagents. Sodium periodate (Meta, G. F. Smith & Co.) was stored in a 10^{-2} M solution in distilled water. From this quantities were added to sea water so as to obtain the appropriate concentration range 10^{-5}–10^{-4} M. Direct addition of the 10^{-2} M periodate to a stirred egg suspension proved to cause no injury. This is often a convenient method when one wishes to improve eggs showing a deficient membrane formation. Of the other oxidants used in this work also 10^{-2} M solutions were prepared that were diluted with sea water as described for periodate. All storage solutions were kept in refrigerator and often renewed. The pH was controlled with a Beckman pH-meter with glass electrode. The percentage figures given below refer to counts of about 125 eggs in each test within an experiment.

RESULTS

The action of some different concentrations of sodium periodate on fertilization and development is described in Exp. 1.

Exp. 1 (Naples, April 1953).

A material of eggs of *Paracentrotus lividus* was inseminated; only 15–20 per cent of the eggs were fertilized. Samples of unfertilized eggs of this material were subjected to the following solutions for 30–40 minutes.

a. $2 \cdot 10^{-5}$ M sodium periodate in sea water.
b. $4 \cdot 10^{-5}$ M sodium periodate in sea water.
c. $1 \cdot 10^{-4}$ M sodium periodate in sea water.

The eggs were thereafter washed and inseminated in normal sea water. On examination 90 minutes after insemination the following percentages of fertilization were found:

a: 27, *b:* 53, and *c:* 100.

Within the concentration range, $2 \cdot 10^{-5}$–$1 \cdot 10^{-4}$ M the percentage of fertilization was proportional in this experiment to the concentration of periodate.

After 43 hours the following was recorded:

I (control). A minority of the eggs had developed into swimming radially symmetrical larvae.

a. More pluteus-like larvae than in I, but of rudimentary organisation; undeveloped arms and apical part.

b. Pluteus-organisation somewhat more advanced than in a_1 but still subnormal.

c. Well developed plutei with arms, elongated apical part and fully developed oral field, sometimes with a stomodeum.

The pretreatment of the eggs with periodate improved not only the fertilization but also the differentiation capacity of the eggs; subsequent to the pretreatment with the highest concentration used the development was normal.

The concentration range of periodate within which it gave improvement of fertilization and development varied somewhat in different experiments. In some experiments (February 1954) a $2 \cdot 10^{-5}$ M solution of periodate gave the best results; after pretreatment with $4 \cdot 10^{-5}$ M solution the improvement of the membrane elevation was as great as in the lower concentration but the pluteus larvae exhibited some slight signs of injury.

As described in a previous paper (20) the development of *Paracentrotus* and particularly of *Arbacia* eggs in the season 1953 frequently resulted in the formation of radially symmetrical larvae, see above Exp. 1. When the experiments were repeated in February 1954 normal development was obtained. It occurred, however, that the larvae developing from untreated eggs possessed shorter arms than larvae originating from eggs which had been subjected to $2 \cdot 10^{-5}$ M periodate prior to fertilization. The improvement of development was thus not as striking as in Exp. 1.

It was of interest to inquire whether periodate is able to improve the membrane formation after the fertilization has occurred. As shown in Exp. 2 this is not the case.

Exp. 2. (Naples, May 1953).

Eggs of *Arbacia lixula* were subjected to periodate ($8 \cdot 10^{-5}$ M):

a. 6 minutes before insemination;
b. 1 minute after insemination;
c. 1.5 minutes after insemination;
d. 2 minutes after insemination.

After 8–10 minutes the following was observed:

a. A refractile membrane appeared in all eggs.
b. There was a tendency to local elevation of the membrane in 9 per cent of the eggs. The remainder of the eggs had a closely adhering membrane.
c. Similar to *b*) but tendency to local elevation in only 2–3 per cent of the egg.
d. Similar to *c*).

Control eggs fertilized in sea water showed the same low percentage of local membrane elevation as in *c*) and *d*).

In some experiments the action of sodium periodate and sodium iodate were compared, see Exp. 3.

Exp. 3 (Naples, June 1953).

Eggs of *Arbacia lixula* were subjected to the following media:

a. 5 ml sea water;
b. 5 ml sea water with 10^{-4} M sodium periodate;
c. 5 ml sea water with 10^{-4} M sodium iodate.

The eggs were inseminated 15 minutes after transfer to the media.

The following observations were made 4–5 minutes later:

a. 100 per cent fertilization with closely adhering somewhat granular membrane.
b. 100 per cent well elevated smooth refractile membranes.
c. membrane as *a*.

About 60 minutes after insemination samples of eggs were transferred from *a–c* to 0.09 per cent lysozyme in sucrose solution.

The following observations were made after ca. 10 minutes.

a. Strong precipitation of the jelly coat. Its outline was strongly folded.
b. The jelly coat appeared as a thin seam with no folds; its ground substance granular.
c. Strong precipitation and folding of the jelly coat as in *a*.

These observations indicate that the jelly coat is still highly polymerized in *a* and *c*. The folded appearance corresponds to a mucin clot (Meyer [11]), whereas the granular appearance in *b* indicates a depolymerization. The depolymerizing effect of periodate on the jelly substance was also demonstrated by viscosimetric estimation (16). The thinness of the jelly coat in *b* after precipitation indicates that part of its material had gone into solution. Subsequent to treatment with periodate the jelly coat dissolved gradually see below p. 332.

It thus follows that periodate but not iodate treatment depolymerizes the jelly coat and this is parallel to the effect of the reagents on the membrane elevation.

Exp. 3 continued.

After 45 hours the following records were made:

a. Radially symmetrical larvae almost without pigment cells. Endoderm underdeveloped.
b. Bilaterally symmetrical plutei with well developed arms and stomodeum.
c. Radially symmetrical larvae as in *a*.

The larvae in *a* and *c* hatched in a normal way from the membranes. The difference can thus not be due to any constraint on the larvae. It is obvious that the pretreatment with periodate switches the development into a normal course.

In Exp. 4 the effect of equimolecular solutions of sodium periodate and hydrogen peroxide were compared.

Exp. 4 (*Naples, April 1953*).

Eggs of *Paracentrotus lividus* were transferred to three different beakers containing the following media:

I. 50 ml sea water;
II. 50 ml sea water with 4.10^{-5} M sodium periodate;
III. 50 ml sea water with 4.10^{-5} M H_2O_2.

Small samples of these eggs were transferred after ten minutes to

a. 2 ml sea water, eggs from I;
b. 2 ml sea water, eggs from II;
c. 2 ml sea water, eggs from III.

The eggs in *a* and *c* were often slightly elongated in one direction, indentated or angular whereas those in *b* were spherical or almost spherical. The jelly coats were rather condensed in *a* and *c*, but strongly swollen in *b*.

After about 10 minutes *a–c* were inseminated:

a. The membrane elevation started 80 seconds after insemination.
b. The membrane elevation was in full course already 50–60 seconds after insemination. After 3 minutes the membrane was more elevated in *b* than in *a* and *c*.
c. Membrane elevation as in *a*.

About 20 minutes after the insemination the following observations were made:

a. The egg surface has grown smooth; the membrane was elevated. Numerous granules were present in the perivitelline space. They were often arranged in rows and exhibited a certain limited Brownian movement as if they were embedded in strands extending between the egg surface and the membrane.
b. Membrane more elevated, smoother and more refractile than in *a*, no granules in the perivitelline space.

c. Membranes lower and thinner, less refractile than in *b*. They often contained granules. There were granules present also in the perivitelline space.

Thus the pretreatment with hydrogen peroxide does not bring about the same improvement of the membrane formation as sodium periodate. In several experiments the retardation of the membrane was found to amount to about 12 mμ in the periodate pretreated *Paracentrotus* eggs. In the hydrogen peroxide pretreated eggs the retardation was considerably lower, amounting to 6–8 mμ.

Exp. 4 continued.

After 21–22 hours the state of development was the following.

a. Somewhat irregular gastrulae of bilateral form (prisms); skeleton in the form of small triradiate bodies.

b. Regular gastrulae of bilateral form (prisms) with well developed skeleton rudiments. The cells of the primary mesenchyme arranged in a more regular way than in *a*, ectodermic epithelium smoother and more expanded than in *a*.

c. Gastrulae with a tendency to radial symmetry. The primary mesenchyme is rather irregular, the development of the skeleton is strongly inhibited. At most minute rudiments are visible. Ectodermic epithelium less flattened than in *b*. Mesenchyme with a certain tendency to cytolysis.

There is thus an unmistakable aftereffect of the pretreatment with hydrogen peroxide in the form of inhibition or injury. The eggs pretreated with periodate, on the other hand, gave a better development than the non pretreated control.

The eggs of *Arbacia lixula* reacted in a similar way as the *Paracentrotus* eggs to hydrogen peroxide. The pretreatment with this reagent in the concentration $2–4 \cdot 10^{-5}$ M did not bring about a membrane elevation, whereas this was the case when eggs from the same material was subjected to pretreatment with periodate.

Hydrogen peroxide did not promote the swelling of the jelly coat as does treatment with periodate.

Exp. 5 (Naples, April 1953).

Non fertilized eggs of *Arbacia lixula* were subjected to periodate on hydrogen peroxide (2×10^{-5} M) in sea water for a prolonged period. There was an immediate swelling of the jelly coat in periodate; after 30–40 hours the jelly coat was practically dissolved, whereas at this time no swelling had occurred in the hydrogen peroxide - sea water medium. Samples of eggs were subjected to the modified mucin clot test (transfer to 0.09 per cent lysozyme in isotonic sucrose solution, see above under Exp. 3). The eggs subjected to periodate exhibited a granular thin membrane indicating dissolution and depolymerization until finally no jelly coat could be demonstrated due to its complete dissolution. The eggs subjected to hydrogen peroxide,

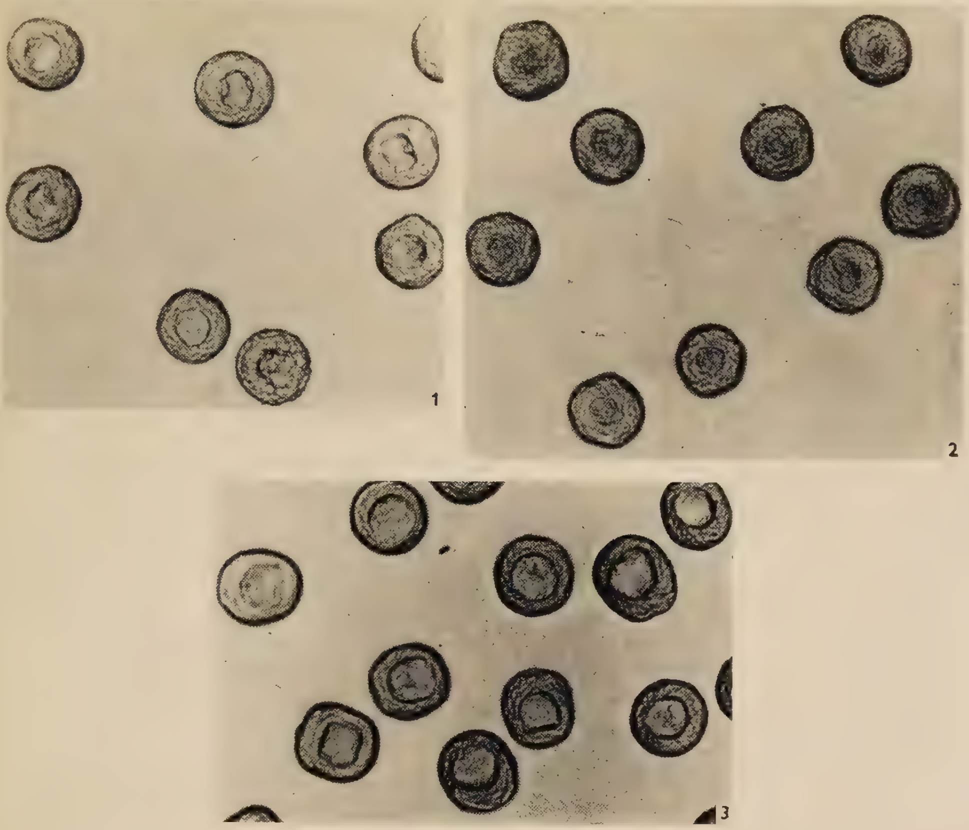

Fig. 1–3 refer to Exp. 6, blastulae *1* from control eggs in sea water, *2*, from eggs fertilized in presence of 2×10^{-5} hydrogen peroxide, *3*, from eggs fertilized in presence of 2×10^{-5} periodate in sea water.

on the other hand, exhibited even after 40 hours a strongly folded jelly coat when exposed to lysozyme in sucrose solution. Obviously no dissolution or depolymerization occurred subsequent to the action of hydrogen peroxide.

It is of interest that the size of the blastocoel is also influenced by the oxidizing agents. There is a striking parallelism between the effect of the reagents on the jelly coat and on the blastocoel.

Exp. 6 (Naples, February 1954).

Non fertilized eggs of *Paracentrotus lividus* were transferred to

a. Sea water;
b. Hydrogen peroxide 2×10^{-5} M in sea water;
c. Periodate 2×10^{-5} M in sea water.

Two minutes later the eggs were fertilized. The early events occurred as described in Exp. 4. The blastulae were studied 18 hours after fertilization:

a. Relatively thickwalled hatched blastulae with a rather small blastocoel, see Fig. 1.
b. Blastula with still thicker walls and narrower irregularly shaped blastocoel, see Fig. 2.
c. Blastulae with large regularly shaped blastocoel. The difference between the animal and vegetal pole of the blastula is more conspicuous than in *a* and *b*, see Fig. 3.

After 65 hours more than 50 per cent of the larvae in *c* were fully developed plutei and the remainder was in the late prism stage. In *a* the development was retarded in comparison to *c*, only larvae in prism stage were observed and these deviated often somewhat from the normal. In *b* the swimming larvae were in the same stage as in *a* but a great number of larvae was on the bottom of the dish. A loosening of the cell continuity had occurred already in the early gastrula stage.

Addition of periodate ($4 \cdot 10^{-5}$ M) to hydrogen peroxide ($4 \cdot 10^{-5}$ M) pretreated eggs of *Paracentrotus lividus* (Naples, April 1953) made the eggs more spherical and improved the membrane elevation. The further development was, however, more pathological than after hydrogen peroxide treatment alone.

To test the effect of potassium ferricyanide on the fertilization of the sea urchin egg experiments of the following type were carried out.

Exp. 7 (*Naples, June 1953*).

Eggs of *Arbacia lixula* were transferred to the following media.

a. 5 ml sea water;
b. 5 ml sea water with $4 \cdot 10^{-5}$ M potassium ferricyanide;
c. 5 ml sea water with $3 \cdot 10^{-5}$ M sodium periodate.

After 20 minutes insemination was carried out in these media.

Examination after further 10–15 minutes:

a. > 98 per cent fertilization with closely adhering membranes;
b. 33 per cent fertilization with closely adhering membranes;
c. 100 per cent fertilization with elevated refractile membranes.

After 45 minutes the eggs in *a–c* were washed and thereafter transferred to normal sea water. Here the eggs in *b* were reinseminated; now almost 100 per cent of the eggs were fertilized with closely adhering membranes.

Even in the low concentrations used ferricyanide inhibited the fertilization to a great extent, but the effect was reversible. No improvement of the membrane formation was caused by subsequent exposure to sodium periodate. The motility of the spermatozoa was not disturbed by the ferricyanide; on the contrary it seemed to be enhanced.

In a similar experiment as the above, ferricyanide was added to an egg suspension (final concentration: $4 \cdot 10^{-5}$ M); 15 minutes later periodate was added (final concentration: $3 \cdot 10^{-5}$ M). Upon insemination only 3 per cent of the eggs were fertilized. The jelly coat underwent a considerable swelling. In a parallel sample of the same egg material the order was reversed so that periodate was added first and ferricyanide 15 minutes later. No fertilization occurred, but a strong swelling of the jelly coats was noted. After washing and transfer to normal sea water reinsemination was carried out; this resulted in close to 100 per cent fertilization with closely adhering membranes. The ferricyanide thus suppressed the improving effect of periodate upon the membrane elevation also after transfer to normal sea water. This was the case even if the eggs were pretreated with ferricyanide and then transferred to normal sea water and thereafter subjected to periodate as the following experiment shows:

Exp. 8 (*Naples, June 1953*).

Eggs of *Arbacia lixula* were transferred to the following media.

I. 5 ml sea water;
II. 5 ml sea water with 4.10^{-5} M potassium ferricyanide.

After 12 minutes eggs were washed and transferred from II to

a. 1 ml sea water;
b. 1 ml sea water with extract from the granular fraction of egg homogenate (N-content 110 μg per ml, see (18));
c. 1 ml sea water with 3.10^{-5} M sodium periodate.

Here the eggs were immediately inseminated. At the same time I and II were inseminated.

Examination after 10–15 minutes.

I. 100 per cent fertilized with closely adhering membrane.
II. 68 per cent non fertilized, 32 per cent with closely adhering membranes.

a. >98 per cent fertilized with closely adhering membrane.
b. 100 per cent fertilization with elevated refractile membranes; jelly coats swollen.
c. 100 per cent fertilization with closely adhering membranes.

Two hours after insemination the media were diluted with sea water. In this way the extract was diluted about 1:6. The same was the case with the periodate which, however, is of no relevance as the periodate is rapidly broken down, particularly in presence of eggs with jelly coats.

After 41–43 hours the following observations were made:

I. Free swimming larvae of the radially symmetrical type.
II. No development had occurred.

a. As I.
b. Bilateral free swimming larvae of pluteus type.
c. Development rather similar to that in *b*.

The observations were repeated 60 hours after the insemination. The earlier observations (after 41–43 hours) were confirmed with addition of numerous details. Even if the larvae in *c* are bilateral a certain reminescence of the radial type remained. This was particularly obvious in the most animal part which sometimes had the appearance of a lobe. The normal shift of the animal pole in oral direction had failed to occur. Despite this well developed anal arms were present. The apical part was typically developed with flattened epithelium and the apical parts of the skeleton showed the characteristic enlargement with formation of numerous spine-like outgrowths. In many larvae, however, the pluteus organisation is more perfect with a typical oral field surrounded by a continuous ciliary band. In *b*, on the contrary, there was sometimes a deviation from the normal which consisted in a certain reduction of the animal differentiation. This is due to the presence of dilute extract which was shown to have this effect (20).

The results presented show that pretreatment with ferricyanide prevents the improving effect of periodate on the membrane elevation. Conversely, the anti-radializing effect of periodate revealed itself even subsequent to a pretreatment of the eggs with ferricyanide.

Like the extract of the granular fraction of egg homogenate, glutathion has the capacity of removing the effect of the pretreatment with ferricyanide as experiment 9 shows.

Exp. 9 (*Naples, June 1953*).

Unfertilized eggs of *Arbacia lixula* were transferred to the following media:

I. 1 ml sea water;
II. 1 ml sea water with $1.7 \cdot 10^{-4}$ M potassium ferricyanide.

After 16 minutes eggs were transferred after washing from II to

a. 5 ml sea water;
b. 5 ml sea water with 0.017 per cent glutathione.

Upon insemination the following observations were made.

I. closely adhering membrane;
II. low percentage of fertilization with adhering membrane;

a. closely adhering membrane;
b. separation and elevation of the membrane occurred.

Eggs were transferred from I also to 5 ml sea water with $2 \cdot 10^{-5}$ M periodate. Upon insemination a membrane elevation took place here. The membranes were more elevated and more refractile than in *b*.

After 48 hours the state of development was the following.

I. Larvae with an animal lobe; no arms, undeveloped skeleton; radial symmetry with a certain tendency to bilateralization.

a. Radialized larvae which exhibit also a rather pronounced animalization with enlarged ciliary tuft.

b. Well developed bilateral plutei.

Larvae were raised also from the eggs which were inseminated in presence of $2 \cdot 10^{-5}$ M periodate, see above. They were plutei considerably larger and more vigorous than those in *b.*

In February 1954 some further experiments were carried out with eggs of *Paracentrotus* that when kept in normal sea water developed into bilateral plutei. These eggs were treated in the unfertilized stage for 2–10 minutes with $2\text{–}4 \times 10^{-5}$ ferricyanide. They were then inseminated, 15–40 minutes later transferred to normal sea water and washed with this several times. Like hydrogen peroxide, ferricyanide inhibits the swelling of the jelly coat. The spermatozoa stick to the jelly where they accumulate. The insemination brought about a varying percentage of fertilization; the membranes were closely adhering to the egg surface or showed at most a local elevation. The blastulae (10–11 hours stage) were thick-walled with narrow blastocoel. The developing larvae were of the radially symmetrical type (observations 35–60 hours after fertilization). They exhibited the characteristic animal lobe with cylindrical epithelium. The anal field was surrounded by a folded ciliary band. The skeleton consisted of a number of triradiate spicules, see for further characterization (4) and (20). Bilateral plutei were found both in the control in sea water and in a parallel in which the eggs from the same material were subjected to $2\text{–}4 \times 10^{-5}$ periodate. The time of exposure was exactly the same as in the case of the ferricyanide treatment. The plutei arising from the periodate treated eggs were larger and more fully developed than those in the control.

It is obvious that a rather brief exposure of the uncleaved egg to ferricyanide sufficed to induce a radially symmetrical organisation of the larva that is manifested only in a relatively advanced stage. The developing egg cannot by its own resources overcome the effect of the oxidizing agent.

An extract from *Fucus vesiculosus* inhibits fertilization or at least the membrane elevation, see (21). The inhibitory factor from *Fucus* is called fertilization inhibitor Fucus, FeInh[Fu]. The not highly purified preparation B 6 was used, see (21).

Exp. 10. (Naples, April 1953).

Eggs of *Paracentrotus lividus* were transferred to the following media:

a. 1 ml sea water with 0.01 per cent FeInh[Fu] B6;

b. 1 ml sea water with 0.01 per cent FeInh[Fu] B6 and with $6 \cdot 10^{-5}$ M sodium periodate;

c. 1 ml sea water with 0.01 per cent FeInh[Fu] B6 and with $6 \cdot 10^{-5}$ M hydrogen peroxide.

The eggs were inseminated after 16 minutes in these solutions.

a. Appr. 60 per cent of the eggs fertilized, but under contraction, only a thin closely adhering membrane appeared.
b. 95 per cent with well elevated refractile membrane.
c. Appr. 10–20 per cent of the eggs fertilized with thin closely adhering membranes.

It thus seems that hydrogen peroxide instead of removing the inhibitory effect of FeInh[Fu] enhanced this effect.

After 7 hours spherical blastulae with a rather large blastocoel and an elevated membrane were observed in *b*, whereas the blastula in *a* and *c* were rather compact with no or a narrow blastocoel and no elevated membrane. After 25 hours the eggs have developed into gastrulae in *a–c*. There was no pronounced bilateral symmetry. In the course of the further development, however, the larvae in *b* assumed bilateral symmetry and developed into plutei with rudimentary arms. The larvae in *a* and *c*, on the other hand, remained radially symmetrical with a very small number of pluteus-like larvae.

In a parallel control in pure sea water more than 95 per cent of the eggs were fertilized with elevated membranes; after 26 hours the larva were bilateral gastrulae (prism-stage) and they developed further to rather normal plutei.

In contradistinction to periodate hydrogen peroxide caused no removal of the inhibitory action of FeInh[Fu]; on the contrary, the number of non fertilized eggs increased. Periodate removed also the radializing action of the FeInh[Fu] whereas hydrogen peroxide had no action.

Experiments of this kind were repeated in February 1954 with eggs of *Paracentrotus* which in normal sea water developed into plutei. The more potent preparation of FeInh[Fu], Sup C_4 I, was used. This made only the difference that lower concentrations of the inhibitor were used. Again periodate removed the inhibitory effect of the FeInh[Fu], but hydrogen peroxide was not able to do so. On the whole the latter held true also for ferricyanide; its effect—if any—is weak in comparison with that exerted by periodate.

Experiments were also made to study the effect of iodine—potassium iodide ($1 \cdot 10^{-5}$–$8 \cdot 10^{-5}$ M solutions in sea water). The few tests made showed definitely that iodine had not the improving action on the fertilization characteristic of periodate. Concerning the effect of iodosobenzoate, see (2, 9).

DISCUSSION

The previous data accentuate the unique effect of periodate in improving the fertilization and development of the sea urchin egg. None of the other oxidizing agents exert the same action. Both hydrogen peroxide and ferricyanide impair the fertilization to a variable extent. Iodide and iodate seem to be rather indifferent. There is a striking parallel between the improving action on the fertilization and a swelling effect on the jelly coat. This holds true for a number of other reagents which improve fertilization, see (18, 22). The oxidizing agents which caused no improvement of fertilization did not bring about a swelling of the jelly coat. Nevertheless the state of swelling of the jelly coat is not the decisive factor. The spermatozoa are able to penetrate a jelly coat which has been precipitated by the jelly precipitating factor from the egg (JePptF[E]) or "antifertilizin", see (6, 20).

Moreover, periodate is able to improve membrane elevation even if the jelly coat has previously been removed (8). The most probable explanation is that substances are present in the cortex of the egg which react in a similar way as the jelly coat. These substances constitute the "target" of the periodate action. This special target is, however, inaccessible to the other oxidizing agents used in this research or they influence the target in a different way. Pretreatment of the eggs with potassium ferricyanide interferes with a subsequent action of periodate with respect to the membrane formation. This must not necessarily mean that the oxidizing agents react with the same groups. The oxidation with ferricyanide accentuates the refractory state, whereas the periodate hits some inhibitory group and removes its action.

Periodate is able to eliminate the fertilization inhibitor from *Fucus* (FeInh [Fu]) see (5, 21), whereas hydrogen peroxide is not. The action of ferricyanide in this respect is at most very weak. There is thus a great similarity between the FeInh[Fu] and the natural inhibitor in the egg. This latter is also eliminated by periodate but not by hydrogen peroxide or ferricyanide.

As shown previously (8) periodate has in appropriate concentrations an activating effect on the unfertilized egg. Most significant in this respect are the results obtained by Allen (1). He found that a local application of periodate on the egg surface may start an activation impulse which spreads over the surface of the egg. Other activating agents as hypertonic sea water or bee venom caused only a local change.

Several non reported observations made in the course of this work substantiated the earlier statements regarding the activating action of periodate, whereas none of the other oxidizing agents had this effect.

The action of the different oxidizing agents tested cannot be predicted by their oxidation-reduction potentials. Specific reactions with different systems in the cell are probably the main factor to be considered.

Is it possible to arrive at any concept regarding the nature of the target upon which periodate is acting? Let us remember the statement (8) that anisodiametric eggs very rapidly become spherical if subjected to the action of periodate. This is probably due to the decrease in rigidity of the cortex which Kriszat (7) has shown to occur in periodate treated eggs. It seems probable that polysaccharides or complexes with these are present in a state of high polymerization in the cortex of the anisodiametric egg. A depolymerization of these polysaccharides or complexes subsequent to periodate treatment may give rise to the decrease of rigidity. A support is given to this contention by the demonstration (7) that upon addition of FeInh[Fu] or of chitindisulfate the rigidity of the egg cortex increases.

The decrease in rigidity of the cortex runs parallel to a swelling of the jelly. It has already been pointed out above that periodate causes a strong swelling and a depolymerisation of the jelly substance. Nothing similar occurs when the other oxidizing agents are added.

In presence of hydrogen peroxide or ferricyanide a number of spermatozoa were seen to stick to the jelly coat due to the high rigidity of the jelly substance. The mucin clot test in the modification used in this work (p. 330) also clearly demonstrated the difference between the action of periodate with its depolymerizing effect and that of hydrogen peroxide and ferricyanide which certainly caused no depolymerization. In contradistinction to periodate hydrogen peroxide and ferricyanide cause no sphering of the egg. It is thus improbable that the last named agents like periodate will prove to decrease the rigidity of the egg cortex in the technique used by Kriszat.

Lundblad (10) found that periodate is able to activate one of the proteolytic enzymes present in the egg (E II). According to his interpretation periodate eliminates an inhibitor of polysaccharide nature. This was confirmed by the demonstration that addition of heparin blocks the activity of the enzyme E II. Lundblad's results are apt to give an insight into the mechanism of the action of periodate on the egg. It shows how the agent may be able to remove an enzyme inhibitor and in this way cause an activation or at least what Kriszat (7) called a preactivation.

According to Lundblad the activity of E II very soon subsides. Instead two other proteolytic enzymes, E I and E III, become activated to subside again after about 10 minutes. These enzymes active only for a relatively short period, are possibly able to release certain peptides of importance for the activation and development of the egg. Gustafson *et al.* (3) demonstrated in extracts from lyophilized sea urchin eggs the presence of growth promoting substances acting on *Lactobacillus casei*. These have peptide character and change their character according to the stage of development.

The point of attack of the other oxidizing agents has not been elucidated in this work. As glutathione (Exp. 9 p. 336) has a certain protecting effect subsequent to exposure to ferricyanide this reagent at least partly acts by oxidizing SH-groups. Iodosobenzoate is also known as an oxidizing agent for SH-groups but its action on fertilization is weak, see however (13).

It has been observed that the jelly coat may be tougher in mature eggs of *Psammechinus miliaris* than in the oocytes. This was demonstrated by the different ease with which the spermatozoa penetrated the jelly coat. This may imply that the jelly coat substance after maturation undergoes a certain polymerization which probably reflects a general polymerization also of the polysaccharides in the cytoplasmic surface. The phenomenon of underripeness may correspond to such a state of polymerization of polysaccharides. In a polymerized state these may act as inhibitors by binding proteins including enzymes. A mutual precipitation may occur. Cytoplasmic ripening may be supposed to correspond to a gradual decrease of the polymerization until a certain level is reached representing optimum conditions for fertilization and development. Periodate instantaneously converts the underripe into the ripe state, whereas ferricyanide and hydrogen peroxide confer upon the cytoplasm a state similar to underripeness.

The same effect is obtained in the presence of the fertilization inhibiting factor from *Fucus* in concentrations causing no complete inhibition of fertilization. Possibly also the addition of other substances or contamination of the sea water with unknown substances will act in the same way. The effect on the egg seems always to be a similar one. The fertilization is followed by strong contractions of the eggs; an incomplete membrane formation occurs and granules are present in the perivitelline space. The state of the cytoplasm causing these effects is usually rapidly removed subsequent to exposure to certain low concentrations of periodate.

The effect of periodate on the development is perhaps still more remarkable than its improving action on the fertilization. If the eggs are pretreated in the unfertilized state e.g. for 10 minutes with periodate, the developing blastulae are often larger due mainly to a stronger swelling of the blastocoel, Fig. 3. Conversely, it has been shown that pretreatment with hydrogen peroxide or ferricyanide cause a decrease in size of the blastulae due mainly to the small volume of the blastocoel. This is in keeping with the conception formulated above. The blastocoel contains polysaccharides secreted from the cytoplasm, see Monné and Hårde (12). It seems probable that an increased polymerization will impede the secretion of the substances in question.

A brief exposure of the eggs before insemination to the agent may be reflected in morphogenetic processes displaying themselves in larvae several days old. In a control in sea water the development may proceed rather normally but in the pluteus stage the arms may be shorter than in an other portion of the same material that was subjected to a brief pretreatment with periodate, see above p. 337. The larvae from pretreated eggs exhibited often also a greater vigor than the control larvae. The former were more lively and formed the vertically extended zones of aggregation characteristic of healthy plutei.

Particularly striking was the demonstration of the periodate action in such egg batches of *Arbacia lixula* or *Paracentrotus lividus* which had a tendency toward radialization. By means of a brief pretreatment with periodate the development is corrected. As has been previously shown the radialization is not simply a consequence of the failure of the fertilization membrane to elevate from the egg surface. Also eggs with the closely adhering membrane may develop into normal plutei (20). A striking demonstration of this has been given above. Eggs pretreated with ferricyanide formed a closely adhering membrane, even if they are subjected to periodate before insemination. Nevertheless they developed into rather normal plutei although the development of eggs pretreated with ferricyanide alone resulted in radialized larvae.

The conclusion that periodate acts on a "target" is reinforced by the observations on the developmental effects of the periodate treatment. An analogous result was obtained with ferricyanide. Brief exposure of the unfertilized eggs to this reagent was reflected in the development. In this case, however, radially symmetrical larvae resulted.

SUMMARY

As in previous work (16) sea urchin eggs were pretreated with periodate in low concentrations ($< 10^{-4}$ M in sea water). A considerable improvement of the fertilization follows upon this pretreatment. A higher percentage of fertilized eggs may be found; particularly the rate and extent of membrane elevation increases. After the periodate treatment no granular components are found in the membrane or the perivitelline space and the contraction of the egg is inconspicuous. In appropriate concentrations periodate may also exert an activating effect on non fertilized eggs (8, 16).

The aim of the present paper was to add further details of the periodate action but particularly to compare the effect of periodate with that of a

number of other oxidizing agents. Thus hydrogen peroxide, potassium ferricyanide, iodate, iodine, iodosobenzoate were tested in the same molar concentrations as periodate. The effect of periodate was found to be unique. None of the other reagents tested had a similar improving action on the fertilization.

Periodate exerts a swelling and dissolving action of the jelly coat, whereas hydrogen peroxide or ferricyanide bring about an inhibition of the swelling of the jelly coat. There seems to be a correlation between the swelling effect on the jelly coat and the improvement of the fertilization. Nevertheless the improvement by periodate treatment can also be found in eggs deprived of their jelly coats.

In the presence of ferricyanide (e.g. $4 \cdot 10^{-5}$ M) the percentage of fertilization was low but washing increased the number of fertilized eggs to almost 100 per cent. If the eggs are treated with ferricyanide prior to addition of periodate no improvement of the elevation occurred. The same result was obtained if the order of treatment was reversed (see further Exp. 8).

According to previous work (5, 21) periodate removes the action of the fertilization inhibitor extracted from *Fucus vesiculosus* (FeInh[Fu]). None of the other oxidizing agents so far tested removed the inhibitory effect of FeInh[Fu].

The treatment of the eggs before or during fertilization was often reflected in advanced stages of the development. Pretreatment with periodate even before fertilization removed a tendency to radialization which was often found to prevail in the spring of 1953. But also if the control eggs developed into four-armed plutei eggs pretreated with periodate (e.g. $2 \cdot 10^{-5}$ M) gave more vigorous larvae with a greater growth rate of the arms.

A brief exposure to ferricyanide (e.g. $2 \cdot 10^{-5}$) on the other hand seems to cause a radialization of the larvae. Exposure to glutathione or to egg extract subsequent to the treatment with ferricyanide at least partly removes the effect of this reagent.

The apparent specificity of the periodate action is probably due to its reaction with carbohydrates present in the egg surface as polysaccharides. In a polymerized state these seem to act as inhibitors both in fertilization and development. The periodate exerts its action by removing the inhibition.

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INCORPORATION OF LABELED METABOLITES IN CHORIOALLANTOIC MEMBRANES INFECTED WITH INFLUENZA VIRUS

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The chorioallantoic membrane of chick embryos presents a very favorable cellular substrate for various kinds of viruses. During the last few years, it has been used in a number of experiments pertaining to the metabolic interrelationship between viruses and host cells. It has been shown, particularly by Ackermann *et al.* (1, 2, cf. 8), that the metabolic activity of the host cells is a factor of supreme importance affecting virus multiplication in the membrane.

In the case of influenza virus it has been observed that a more rapid uptake of P^{32}-phosphate into the chorioallantoic membranes of de-embryonated eggs occurs after infection (4). It has been shown, particularly in the case of bacterial viruses (3), that virus infection may induce changes in the metabolic constitution of the host cell. It thus seemed of interest to examine more in detail the incorporation of labeled metabolites into chorioallantoic membranes after infection with influenza virus.

EXPERIMENTAL

White leghorn eggs, on the 13th day of incubation, were used in the experiments.

The chorioallantoic membranes were infected with influenza virus either *in vivo* or *in vitro.*

The infections *in vivo* were performed by injecting into the allantoic cavity of the eggs approximately $10^{8.5}$ ID (cf. 5) of influenza virus A (PR 8) in the form of allantoic fluid, diluted with an equal volume of saline containing 100 μg penicillin and 100 I.U. of streptomycine per ml. In order to attain a rapid and even distribution of the injected virus particles over the inside surface of the membranes, the eggs were placed in a roller drum, turning at a rate of two revolutions per minute and with an inclination of 45°. The rotating drum was kept in an incubator at 37° C. After 30 minutes, the eggs were transferred to an ordinary egg incubator (37° C). At different intervals after infection, eggs were removed from the incubator and circles of 25 mm radius were drawn on the shells (on the side of the eggs, where the chorioallantoic membranes were best developed). The eggs were opened, and the shells with adhering membranes were cut along the circle lines. The membrane circles were removed from the shells, and were carefully washed in Simm's solution (as modified by Ackermann (1)). (P-free Simm's solution in the P^{32}-experiments.) They were then transferred to conical flasks with 8 ml of Simm's fluid, containing the isotopic component. The flasks were supplied with a gas mixture of 95 per cent O_2 and 5 per cent CO_2, and were shaken in a water-bath at 35°C. In the experiments with labeled amino acids, the membranes were kept in the isotopic medium for 60 minutes. In the experiments with P^{32}-phosphate they were generally treated with isotope for 40 minutes.

In a number of experiments, membrane circles taken from non-infected eggs were shaken *in vitro* for 30 minutes in Simm's medium containing approximately $10^{8 \cdot 5}$ ID of influenza virus per membrane circle. The membranes were then washed, and were shaken in a large flask with modified (1) Simm's medium, which was changed every hour. At intervals, membranes were transferred to isotopic Simm's medium, as already described. Non-infected membranes, treated in the same way, were used as controls.

After the isotope treatment the membrane circles were carefully washed in cold Simm's medium, containing a large excess of unlabeled isotope carrier. The membranes were treated with ice-cold 5 per cent trichloroacetic acid (TCA), lipid solvents, and 5 per cent TCA at 90°C (12). Unlabeled carrier in large excess was added at the final extraction in cold 5 per cent TCA. The isotope contents of the fractions were determined in the conventional manner. In the cold TCA-fraction of the P^{32}-experiments, the activities of the acid-labile phosphate esters and of the non-labile phosphate esters were determined according to the method of Martin and Doty (11), as modified by Lindberg and Ernster (10).

RESULTS AND DISCUSSION

The isotope incorporation values of different membranes showed a considerable variation. This is probably largely due to variations in the thickness and consistency of the membrane.

On the whole, the rate of incorporation of N^{15}-glycine, N^{15}-DL-alanine and C^{14}-glycine into the membrane proteins increased continually after infection (Fig. 1). A maximum generally occurred after 6–9 hours. The rate of incorporation then decreased and finally attained a similar or even lower level than before infection. In membranes, which had been infected *in vitro* (both sides of the membranes being infected), the peak values generally occurred earlier (Fig. 1 C).

It was first believed that the increased rate of isotope incorporation into the proteins of the infected membranes was mainly due to a temporarily intensified protein metabolism in the host cells during the course of the primary cycle (7) of virus development. After further study it became evident that other factors were of greater importance.

In experiments with C^{14}-glycine it was observed that the specific activity of the nucleic acid fraction at different times after infection closely paralleled that of the proteins. This close parallelism between different fractions was particularly striking in experiments of a similar kind performed with P^{32}-phosphate. The time course of phosphate incorporation in different phosphate fractions of the membranes was therefore studied (Fig. 2). It became evident from these experiments that the equilibrium between the acid-labile phosphate of the cells and the orthophosphate in the medium was established very slowly, and was particularly delayed in the non-infected membranes. This suggested that the transport of the isotope between the medium and the interior of the cells was restricted by some sort of permeability barrier.

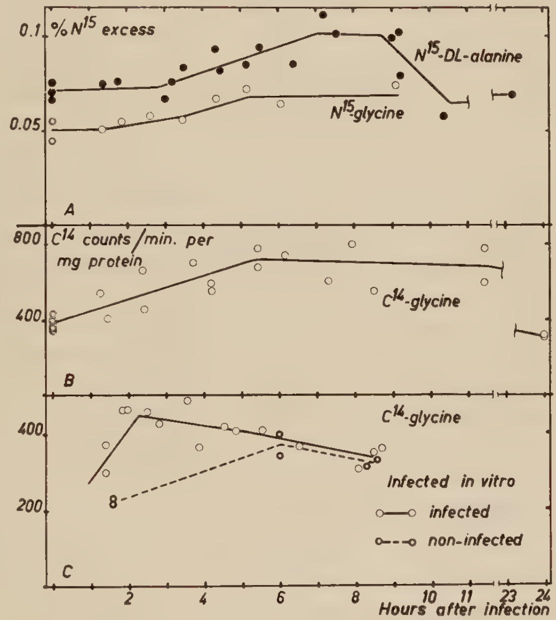

Fig. 1. Rate of incorporation of labeled amino acids into the proteins of chorioallantoic membranes at different times after infection. Each membrane circle had been incubated for 60 minutes in a medium containing the labeled amino acid.

It seemed possible that this permeability barrier could be due to a limited capacity of an enzymatic transport mechanism in the surfaces of the cells. If this were the case, the rate of isotope incorporation should be very susceptible to dilution of the isotope with non-labeled carrier (cf. 9). In some experiments non-infected membranes were treated with P^{32}-phosphate which had been diluted with increasing amounts of non-isotopic carrier-phosphate. It was found (Table I) that the effect of the isotope dilution on the rate of isotope uptake into the intracellular, esterified phosphate fractions was considerably less than could be accounted for by the isotope dilution of the orthophosphate in the medium. These results suggested that it is not a limited capacity of the active transport mechanism in the cell surfaces, which is mainly responsible for the restricted permeability of the membranes, but instead passive diffusion factors, probably located in the intercellular space.

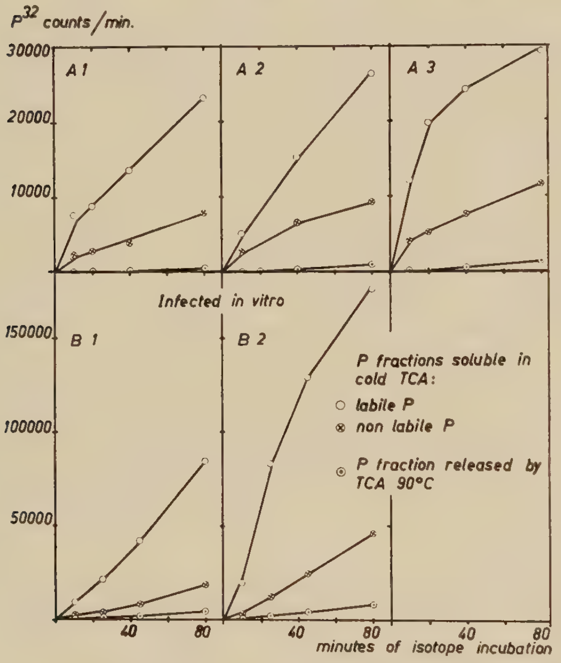

Fig. 2. Chorioallantoic membranes at different stages of infection treated for various lengths of time with P^{32}-phosphate. Isotope uptake into different esterified phosphate fractions.

Series A: Infection *in vivo*. *A 1:* Non-infected membranes. *A 2:* Membranes removed from infected eggs 20 minutes after infection. *A 3:* Membranes removed 5 hours after infection.

Series B: Infection *in vitro*. *B 1:* Non-infected membranes kept in Simm's medium for 50 minutes. *B 2:* Membranes kept for 50 minutes in Simm's medium containing $10^{8.5}$ ID of influenza virus per membrane.

As is well known, the chorioallantoic membrane has a rather loose and mucous structure. Since it has often been postulated that influenza viruses are endowed with mucolytic activities (cf. 6), it seemed possible that the increased rate of diffusion of the isotopic compounds into the infected membranes could be due to a mucolytic loosening of the membrane structure. In that way, a more rapid communication between the isotopic medium and the interior cells of the membrane might be established. In order to decide whether the increased isotope incorporation values of the infected membranes could be explained on the basis of a mucolytic action of the viruses, a number of experiments were performed, in which non-infected membranes were treated *in vivo* or *in vitro* with different concentrations of purified hyaluronidase (Leo). These experiments were carried out in the

Table I

Chorioallantoic membranes incubated for 40 minutes in 8 ml of modified Simm's medium containing P^{32}-phosphate, diluted with different amounts of non-labeled carrier-phosphate.

Amounts of carrier phosphate per sample (μg)	Sp. activity of TCA-soluble phosphate fractions (counts/min/mg protein)	
	Acid-labile P	Non-labile P
10	37,000	10,500
20	37,000	6,500
60	29,500	8,500
260	12,500	4,500

Table II

Effect of hyaluronidase treatment on the uptake of P^{32}-phosphate into chorioallantoic membranes. Each value represents two different membranes. Hyaluronidase solution (H) contained 100 VRU/ml. Time of isotope treatment 40 minutes.

	Sp. activity (counts/min/mg protein) of		
	Cold TCA-fraction		90 °C TCA-fraction
	Acid-labile P	Non-labile P	
Untreated membranes	96,300	14,400	5,400
Membranes treated *in vivo* with:			
0.2 ml H for 1 hour	152,000	26,400	7,300
0.5 ml H for 1 hour	144,000	28,200	6,200
0.5 ml H for 3 hours	178,000	47,000	10,600
Membranes treated *in vitro* with 0.3 ml H per ml medium:			
1 hour	111,000	27,100	7,200
2 hours	146,000	32,300	8,200
Untreated membranes kept in Simm's medium for 3 hours	128,000	20,300	6,700

same way as the virus experiments. Solutions of the enzyme (5-100 VRU per ml) were injected into the allantoic cavities of chick embryos or were added to media in which membrane circles were incubated *in vitro*. As is shown in Table II, the effect of hyaluronidase on the isotope uptake of the membranes was very similar to the effect of influenza virus. It seems very likely, therefore, that the promotion of isotope uptake into chorioallantoic membranes by influenza virus is largely caused by a mucolytic action of the virus similar to the action of hyaluronidase.

As a rule, non-infected membranes kept for several hours in Simm's medium took up isotope from the medium more rapidly than membranes which had just been removed from the eggs (Fig. 1 C, Table II). This may be due to a gradual loosening of the intercellular mucous structure of the membranes under the influence of the medium. At the same time, the membranes became increasingly sticky. In membranes infected *in vitro* with influenza virus the stickiness was less pronounced, probably because the mucous jelly is gradually dissipated.

SUMMARY

Chorioallantoic membranes, which had been infected *in vivo* or *in vitro* with large doses of influenza virus A PR8 (about 2 virus particles per surface cell), were incubated at different intervals after infection with N^{15}-glycine, N^{15}-DL-alanine, C^{14}-glycine, or P^{32}-phosphate. The rate of isotope incorporation increased continually and generally reached a maximum 6–9 hours after infection.

It was shown that the increased rate of isotope uptake was mainly, if not entirely, due to an increased intercellular permeability in the membrane. A similar effect was obtained with hyaluronidase. It seems very likely that mucous substances, which can be attacked by influenza virus as well as by hyaluronidase, are involved in the intercellular permeability barrier.

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DIFFERENCES IN COMPOSITION BETWEEN MITOCHONDRIA FROM TWO MOUSE STRAINS[1]

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The existence of the mitochondria as definite cytoplasmic structures with characteristic cytological properties has been known since the classical studies of Altmann (2). But only after methods had been developed to isolate these particles by means of differential centrifugation has it been possible to study their chemical composition and their function in the metabolism of the cell. In the last decade, a large number of investigations (review by Schneider and Hogeboom (26)) has supplied evidence that these structures play an important role in the enzymatic activities of the cell. Many enzyme systems have been found to be concentrated in the mitochondrial fraction, and some enzymes seem to be restricted to it.

It appears to be well established that the enzymatic activity of isolated mitochondria is profoundly influenced by the medium in which they are isolated (25, 8, 10, 11). It should therefore be expected that changes in their intracellular environment may also affect their structure and function. Actually, it has been demonstrated that environmental factors such as low temperature and, to a lower degree, starvation may cause strong morphological alterations in the liver mitochondria of amphibians (22).

One of the factors which is known to affect intracellular chemical constitution are the genes (4). It may therefore be expected that the activity and structure of mitochondria may be influenced by genetic differences. It was accordingly attempted to investigate whether differences between the mitochondria of different strains of the same species can be demonstrated.

As an organism for these investigations the mouse was chosen, because on one hand it is well analyzed genetically and a large number of genetically homogeneous strains are available. On the other hand it is large enough so that sufficient mitochondrial material for analysis can be obtained from a single organ of a single individual. This is necessary since it is well known that the mitochondria obtained from different organs of the same organism

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may show different composition and properties (24, 28, 1). The present paper is an attempt to demonstrate differences in chemical composition between mitochondria isolated from the livers of mice belonging to two different inbred strains.

MATERIALS AND METHODS

The mice used belonged to the highly inbred strains BALB/Ci and C57 Black. Only males were employed. The animals varied considerably in age. They were starved for 15–18 hours before being sacrificed.

Liver mitochondria were isolated according to the method of Hogeboom, Schneider and Palade (12). The livers of the mice were perfused with ice-cold saline through the hepatic portal vein. After being excised, they were homogenized in 10 volumes .88 molar sucrose with a Potter-Elvehjem homogenizer consisting of a glass test tube and a fitted lucite pestle, and the material was pressed through several layers of cheesecloth to remove connective tissue. The homogenate was layered on top of 2 ml .88 molar sucrose in a centrifuge tube and centrifuged in a clinical centrifuge at 600 g for 10 minutes, in order to remove nuclei, unbroken cells and debris. This process was repeated twice. The supernatant was centrifuged in a Servall SS2 angle centrifuge at 24,000 g for 20 minutes. The sediment was resuspended in .88 molar sucrose and centrifuged a second time at 24,000 g for 20 minutes. The sediment, if suspended in sucrose, consisted microscopically of a homogeneous suspension of rod-shaped elements which stained with Janus Green. After the washing, the sediment was suspended in cold 0.85 per cent saline for analysis. The homogenization and all handling of the preparations were carried out in an ice bath. The centrifuge used in the removal of the nuclear fraction was kept in a refrigerator. The bearings of the Servall centrifuge were cooled with running ice water. Nevertheless, the temperature of the supernatant rose to 10–15° C at the end of the centrifugation at 24,000 g.

In the mitochondrial suspensions prepared in this manner, nitrogen was determined by the Kjeldahl method and phosphorus after the procedure of Fiske and SubbaRow. All determinations were carried out in duplicate.

RESULTS

The difference in P/N ratio between the two strains. The results of our analyses for 76 BALB and 75 C57 males are represented graphically in Fig. 1. The results are expressed as mg phosphorus/g nitrogen. The preparations from both strains show considerable variability. It can, however, be seen that there is a tendency for the preparations from C57 animals to show slightly higher P/N ratios than the BALB mitochondria. This is expressed by the fact that the means of the two distributions are different, and that this difference is significant at the 1 per cent level. The means are 96.26 ± 0.96 mg P/g N for BALB, and 102.03 ± 0.65 mg P/g N for C57 ($t = 4.977$, $df = 150$, $P < 0.01$).

The values for P/N ratio of the mitochondria were actually obtained in 31 different isolations in each of which a number of preparations from both strains were prepared simultaneously. It is therefore possible to subject the total material of 151 values to an analysis of variance. The result is given in Table I.

The results bear out the previous conclusion that there exists a highly significant difference between the two strains, since the variance due to strains is considerably higher than the residual variance ($F = 45.7$ for 1 and 119 degrees of freedom, $P < .01$). But in addition there exists a highly significant variation between the different experiments ($F = 8.42$ for 30 and 119 degrees of freedom, $P < .01$). In other words, the variability from experiment

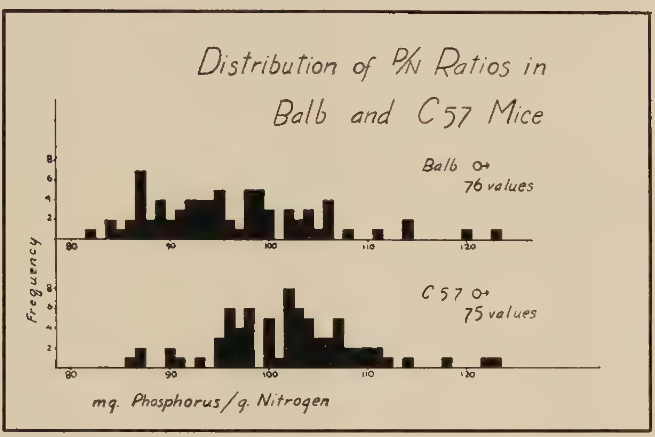

Fig. 1. Distribution of values for phosphorus/nitrogen ratio for 151 mitochondrial preparations from the livers of male BALB/Ci and C57 Black mice (mg P/g N).

TABLE I

Analysis of variance of P/N ratio of liver mitochondria from male mice.

	Sum of squares	Degrees of freedom	Estimate of variance
Between experiments . .	6,943.42	30	231.45
Between strains	1,258.54	1	1,258.54
Residual	2,082.85	119	27.50
Total	10,284.81	150	

TABLE II

Mean P/N ratios, in mg P/g N, for mitochondria isolated in individual experiments from BALB and C57 males.

Experiment	BALB	No.	C57	No.	Difference (C57–BALB)	
1	96.50	1	107.00	1	10.50	
2	106.40	1	110.90	1	4.50	
3	86.71	8	95.44	8	8.73	
4	86.60	4	90.48	4	3.88	
* 5	92.45	4	100.78	4	8.33	
6	94.50	4	101.30	4	6.80	
7	96.95	4	101.60	3	4.65	
8	100.75	4	107.83	4	7.08	
* 9	89.65	2	98.65	2	9.00	
*10	97.70	2	103.00	2	5.30	
11	103.20	2	109.60	2	6.40	
*12	105.00	2	107.15	2	2.15	
13	97.70	2	99.20	2	1.50	
*14	93.70	2	96.20	2	2.50	
15	95.40	2	98.00	2	2.60	
*16	121.60	2	122.50	2	0.90	
17	91.90	2	99.55	2	7.65	
18	97.35	2	100.75	2	3.40	
19	111.25	2	107.80	2		−3.45
20	106.80	2	112.05	2	5.25	
21	99.25	2	109.60	2	10.35	
22	94.50	2	103.90	2	9.40	
*23	93.10	2	101.85	2	8.75	
*24	90.50	2	99.10	2	8.60	
*25	108.05	2	108.50	2	0.45	
26	86.10	2	103.80	2	17.70	
27	104.95	2	104.35	2		−0.60
28	92.90	2	103.95	2	11.05	
29	98.55	2	100.60	2	2.05	
30	97.85	2	97.95	2	0.10	
31	90.00	2	94.30	2	4.30	

to experiment is larger than could be explained by the variability inside experiments. The nature of this significant variation between experiments is not known.

Because of the great variability between experiments, it appears more logical to compare the mean values for the two strains for every one of the

TABLE III

Influence of age on the P/N ratio of the liver mitochondria of BALB/Ci and C57 Bl mice (mg P/g N). (n = number of preparations).

Age in days	1–60	61–120	121–180	181–240	241–300	301–360
BALB . . .	101.13 ± 2.40	94.85 ± 1.30	92.99 ± 1.41	103.50 ± 7.47	104.2	97.95 ± 2.43
n	8	33	16	5	1	10
C57	105.68 ± 2.37	100.36 ± 0.95	101.30 ± 1.60	106.70 ± 4.28		
n	6	38	17	8		

31 experiments. This has been done in Table II. It will be seen that in only 2 out of 31 experiments does the mean value of the BALB samples exceed that of C57. The average difference between the two strains equals 5.48 ± 0.779 mg P/g N ($t = 7.036$, $df = 30$, $P < .01$), a highly significant value.

For most of the animals used, the age was exactly known. It varied considerably, from 41 to 354 days. No systematic attempt has been made to investigate the influence of age on the P/N ratio of the mitochondria. A strong effect of age should, however, be apparent, if the whole material of animals of different ages is compared. The animals were therefore divided into age groups comprising 60 days each, and the means for both strains were calculated. The results are given in Table III.

There is a certain amount of variation between the different age groups. But most of it is certainly due to chance. The high value in both strains in the age group 181–240 days is due to the inclusion in this group of the abnormally high values obtained in experiment 16 (Table II). The other values from animals in the same age group fall into the normal range so that there is no evidence to ascribe the high P/N ratio in experiment 16 to age. The high value in age group 241–300 days (BALB) is based on one preparation only.

There is, however, some evidence that the youngest age group, 1–60 days, has a higher P/N ratio than the older mice. In BALB, the difference between this lowest age group and the next higher one is slightly significant ($t = 2.297$, $P < .05$), that between age group 1–60 days and 121–180 days highly significant ($t = 2.923$, $P < .01$). In C57, the difference between age group 1–60 days and the higher age groups is in the same direction. The difference between the low age group and the next higher one, 61–120 days, is just on the borderline of significance in C57 ($t = 2.086$, $P \sim .05$), while the difference between the low age group and group 121–180 days is insignificant ($t = 1.534$, $P > .1$).

There is therefore presumptive evidence that liver mitochondria isolated from animals 1–60 days old have a slightly higher P/N ratio than those from older animals. Since, however, no systematic investigations for this source of variation have been made, this effect cannot be regarded as proved.

As far as the strain difference is concerned, it cannot be due to a difference in age of the animals used. This can be seen from the fact, appearing in Table III, that for every age group in which material from both strains is available, the P/N ratio is higher in C57 than in BALB. Furthermore, in most of our isolations, care was taken that the animals used in any one experiment were of similar ages. In Table II, experiments in which all animals used were not more than 10 days different in age are indicated by an asterisk. Qualitatively it is seen that in all 9 experiments C57 mitochondria have a higher P/N ratio than BALB mitochondria. Quantitatively, this difference is highly significant (Diff. = 5.11 ± 1.213 mg P/g N, $t = 4.211$, $df = 8$, $P < .01$).

It must therefore be concluded that in spite of the great variation between experiments and a possible variation due to age, a difference in P/N ratio for the mitochondria derived from strains BALB/Ci and C57 Bl is well established.

Chemical nature of the strain difference in P/N ratio. The nitrogen in our preparations comes predominantly from proteins, while the phosphorus is derived from a number of different chemical compounds. In order to describe the difference between BALB and C57 mitochondria more definitely in chemical terms, it was therefore attempted to ascribe the difference in P/N ratio to definite chemical compounds. For this purpose, mitochondrial preparations were subjected to the fractionation procedure of Schneider (23) which divides the phosphorus containing compounds into four fractions: acid soluble, lipids, nucleic acids and proteins.

Five fractionation experiments were carried out. In each one the liver homogenates of three BALB and of three C57 animals were pooled and the mitochondria isolated as described above. The preparations were extracted with 10 ml cold 10 per cent trichloroacetic acid and washed once with 2.5 ml cold 10 per cent trichloroacetic acid. These extracts form the acid-soluble fraction.

The residue was in turn washed with 5 ml 80 per cent alcohol and 5 ml 95 per cent alcohol. It was then extracted three times for 3 minutes each in alcohol-ether 3:1 in a boiling water bath. The alcohol and alcohol-ether extracts constitute the phospholipid fraction.

After extraction of the acid-soluble and phospholipid material, the residue was washed with 2.5 ml 5 per cent trichloroacetic acid. The sediment was extracted for 15 minutes with 5 ml 5 per cent trichloroacetic acid in a boiling water bath, and subsequently washed with 2.5 ml 5 per cent trichloroacetic acid at room temperature. This last trichloroacetic acid extract with its washing constitutes the nucleic acid fraction.

TABLE IV

Distribution of phosphorus in different fractions of mitochondria from BALB and C57 males (mg P per g protein N).

Experiment	Strain	Acid soluble	Lipid	Nucleic acid	Protein	Nucleic acid (Orcinol) (mg RNA per g protein N)
1	BALB	12.0	74.4	33.5	8.8	368.4
	C57	11.5	75.9	39.4	9.0	457.8
2	BALB	11.7	73.5	46.8	7.1	474.7
	C57	12.3	73.3	46.8	7.7	523.5
3	BALB	12.6	68.2	39.9	6.7	417.7
	C57	15.2	70.8	46.2	6.4	511.2
4	BALB	13.7	60.7	33.3	10.9	362.6
	C57	14.0	70.2	35.5	10.4	423.1
5	BALB	16.1	63.9	29.1	9.6	299.2
	C57	19.8	68.5	33.0	9.9	348.2
Mean	BALB	13.2	68.1	36.5	8.6	384.5
	C57	14.6	71.7	40.2	8.7	452.8

The final residue was dissolved in 5 ml 2 per cent NaOH in a boiling water bath. This fraction includes the proteins.

In all four fractions, phosphorus was determined by the Fiske-SubbaRow method. In addition, nucleic acid was determined in the nucleic acid fraction with the orcinol method, since it seems well established that all the nucleic acid in the mitochondria is of the ribose type. The orcinol reaction was standardized against a commercial sample of yeast nucleic acid. Phosphorus determinations of this nucleic acid sample showed that it contained about 15 per cent non-phosphorus-containing impurities. The RNA calibration curve was therefore corrected for the RNA content of the sample, assuming that RNA contains 9.5 per cent phosphorus.

Finally, in the protein fraction nitrogen was determined by the Kjeldahl procedure. The phosphorus and RNA values were expressed as mg per g protein N. The results of these determinations are given in Table IV.

The values obtained in each fraction are quite consistent. If the differences between paired samples of the two strains are compared, it turns out that the differences in acid soluble phosphorus, in lipid phosphorus and in protein phosphorus are not significant. The difference in phosphorus content in the nucleic acid fraction is significant at the 5 per cent level ($t = 3.117$, $df = 4$, $P < .05$). On the other hand, the difference in nucleic acids obtained with the orcinol reaction is highly significant ($t = 7.0126$, $df = 4$, $P < .01$). At least part of the difference in P/N ratio between the two strains is therefore due to substances in the nucleic acid fraction.

An additional fact appears at inspection of the data in Table IV. If the phosphorus content of the nucleic acid fraction is compared to the data obtained with the orcinol method, it is found that in the BALB strain the phosphorus content of the nucleic acids varies from 9.09 (Exp. 1) to 9.86 per cent (Exp. 2) with a mean of 9.48 per cent. In the material from C57, the phosphorus content of the nucleic acids varies from 8.39 (Exp. 4) to 9.48 per cent (Exp. 5), with a mean of 8.89 per cent. This difference is consistent in all paired experiments and statistically significant ($t = 4.941$, $df = 4$, $P < .01$).

This finding might suggest a qualitative difference of the nucleic acids in the mitochondria of the two strains. Such a conclusion seems, however, unlikely in view of the fact that the models of nucleic acid structure require corresponding amounts of sugar and phosphate radicals. The possibility must therefore be considered that the result is due to a higher concentration of non-nucleic acid constituents giving the orcinol reaction present in the "nucleic acid" fraction of C57, or to a larger amount of phosphorus containing contaminants in the "nucleic acid" fraction of BALB mitochondria. At all events, it appears preferable for the time being to ascribe the difference in phosphorus/pentose (orcinol) ratio in the nucleic acid fraction to substances other than nucleic acids. The possibility that this fraction may contain other substances is also suggested by the findings of Huseby and Barnum (13) and of Paigen, Kaufmann and Wood (18) that the nitrogen content of hot trichloroacetic acid extracts from mitochondria is higher than could be obtained from the nucleic acids alone.

Contamination with microsomes. Since microsomes are known to have a higher nucleic acid content than mitochondria, it may be suggested that the difference between the mitochondria of strains BALB and C57 may be due to differential contamination with microsomes. This would not contradict our previous conclusion of a difference in the particulate fractions of our two strains. It would, however, mean that the difference found is actually not a difference in chemical composition but a difference in their ability to adsorb microsomes.

Mitochondria from individual animals were isolated as described above. A sample was removed immediately after the first centrifugation at 24,000 g and P and N determined. The sediment was resuspended in .88 molar sucrose and centrifuged again at 24,000 g. This process was repeated four times, and each time part of the sediment was removed and P and N deter-

TABLE V

Effect of washing on the P/N ratio of mitochondria from BALB and C57 males (mg P/g N).

Number of washings		0	1	2	3	4
Strain	Experiment					
BALB	1	115.5	95.8	94.5	93.8	106.9
		109.3	93.0	94.8	85.8	103.9
	2	110.0	98.6	92.2	95.5	109.7
		107.9	95.9	96.1	109.7	112.2
	3	107.2	98.9	93.1	97.7	103.6
		106.6	100.2	102.3	101.3	105.9
C57	1	113.0	110.0	96.8	107.5	112.5
		122.6	102.7	103.9	96.7	113.4
	2	105.2	96.1	107.6	98.6	96.6
		113.0	108.1	100.0	99.2	123.1
	3	107.6	98.1	97.3	117.0	106.9
		107.7	102.4	96.5	101.6	110.1
BALB	Means	109.4	97.1	95.5	97.3	107.0
C57		111.5	103.1	100.4	103.4	110.4

mined. Three experiments were carried out, each one employing two BALB and two C57 males. The results are tabulated in Table V.

It is seen that in agreement with the findings of Hogeboom, Schneider and Palade (12) the freshly sedimented mitochondrial fractions have a higher P/N ratio than after the first washing. Also, in these freshly sedimented mitochondria, the strain difference is not very clear. This may be attributed to contamination with microsomes. After the first washing, the P/N ratio decreases, and the strain difference becomes evident. In agreement with Hogeboom, Schneider and Palade (12) it was found that the P/N value remains constant through three successive washings. After the fourth washing, however, it was found in 10 out of 12 preparations that the P/N ratio rises again. This may be due to removal of substances lacking phosphorus. A similar effect may be indicated in the data of Hogeboom, Schneider and Palade who found a decrease in succinic oxidase activity after the third washing. No strain difference in the pattern of changes under the influence of washing is apparent from the data in Table V.

On the assumption of stronger adsorption of microsomes on C57 mitochondria, it would be expected that the microsomes would be released more gradually on repeated washing than from BALB mitochondria. Consequently, the evidence in Table V seems to indicate that the difference in P/N ratio of BALB and C57 mitochondria is the result of a real strain difference in the chemical composition of this fraction.

Fractionation of mitochondria. Experiments by Chantrenne (5) and by Paigen and Paigen (19) have suggested that the mitochondrial fraction is a composite of particles which can be sedimented by different centrifugal forces, and which differ in their P/N ratio and their nucleic acid content. More specifically, the particles isolated by higher centrifugal forces are characterized by a higher P/N and RNA/N ratio. If this is the case, our strain difference in mitochondrial constitution may be due either to a qualitative difference in some or all of the fractions, or to an increase in amount of the phosphorus rich smaller particle fractions at the expense of the larger fractions in C57 as compared with BALB. In order to distinguish between these possibilities our mitochondrial fraction was divided by differential centrifugation into three subfractions.

Mitochondria were isolated and washed as described previously. A sample was set aside for the determination of P and N. The remainder was resuspended in .88 M sucrose and centrifuged at 3,000 g for 20 minutes. The supernatant was pipetted off and centrifuged at 6,000 g for 20 minutes. The final supernatant was subjected to a centrifugal force of 24,000 g for 20 minutes. The three sediments obtained in this way were suspended in .85 per cent NaCl and phosphorus and nitrogen determined. The three fractions were called MT1, MT2 and MT3 respectively. On the whole, five separate experiments were carried out. In each single experiment, mitochondrial fractions were isolated separately from four BALB and four C57 males, but one sample from each strain was totally or partially lost so that it cannot be used for comparison. Therefore a total material of 19 preparations from each strain is being compared.

The P/N ratios of the three fractions are given in Table VI, together with the P/N ratio of the total mitochondria. In agreement with the findings of Chantrenne (5) and of Paigen and Paigen (19) it is clearly apparent that the P/N ratio increases with decreasing size of the particles. The differences between the three fractions are highly significant. The difference between the two strains is considerably smaller but highly significant for the total mitochondria. As for the fractions, a higher P/N ratio for C57 as compared with BALB is found only in MT1 and MT2. This difference is highly significant in MT1, just on the borderline of significance in MT2. For the last fraction, MT3, it should be noted that BALB mitochondria seem to have a slightly higher P/N ratio than C57 mitochondria, i.e. the difference

TABLE VI

P/N ratio of different fractions of mitochondria (mg P/g N). The values are means of 19 determinations from each strain.

	Total mitochondria	MT1	MT2	MT3
BALB	95.14 ± 1.35	64.46 ± 0.85	91.96 ± 1.94	149.00 ± 3.63
C57	101.48 ± 1.43	70.10 ± 1.57	99.18 ± 2.91	141.77 ± 1.25
$t(df=36)$	3.230	3.156	2.064	1.881
P	$<.01$	$<.01$	$\sim.05$	$>.05$

TABLE VII

Amount of material present in three mitochondrial fractions, expressed as per cent of total nitrogen. The results are means of 19 determinations for each strain.

	MT1	MT2	MT3
BALB	37.98 ± 2.02	36.46 ± 1.59	25.57 ± 0.96
C57	35.58 ± 1.71	35.95 ± 1.55	28.46 ± 1.14
$t(df=36)$	0.908	0.227	1.944
P	$>.3$	$>.8$	$\sim.05$

is in opposite direction to the difference found in the other two fractions. The difference in MT3 is, however, not significant; at all events there is no evidence for a higher P/N ratio for C57 in this fraction.

The amounts of material present in the three fractions were compared on the basis of their nitrogen content. The nitrogen values obtained in the three fractions from each animal were added up, and the contribution of each fraction was expressed as per cent of the total nitrogen. The mean nitrogen contributions of the three fractions obtained from the 19 determinations from each strain are tabulated in Table VII. It is seen that the differences between the two strains are insignificant for MT1 and MT2. In MT3, C57 contains a higher proportion of total nitrogen than BALB, and since the difference in phosphorus content in this fraction is not significant, the larger amount of material in this fraction would contribute to the increased P/N ratio of strain C57.

From the values summarized in Tables VI and VII, the contribution of every fraction to the total P/N ratio can be calculated. Since we have the relative amounts of nitrogen contributed by each fraction (Table VII) and at the same time the P/N ratio (Table VI), the contribution of each fraction

TABLE VIII

Contribution of different mitochondrial fractions from BALB and C57 males to the total P/N ratio, expressed in mg phosphorus/g total nitrogen. Means of 19 preparations for each strain.

	MT1	MT2	MT3	Total mitochondria (calculated)	Total mitochondria (observed)
BALB	24.48	33.53	38.10	96.11	95.14
C57	25.10	35.66	40.37	101.13	101.48
Difference . . .	0.62	2.13	2.27	5.02	6.34

to total P/N ratio can be calculated by multiplying these two values. This has been done for every single preparation. The means obtained from 19 BALB and 19 C57 animals are shown in Table VIII. In the fourth column, the values of the three fractions are simply added up to give a P/N ratio for the total mitochondria. In the last column, the mean values for the total mitochondria which had been directly determined from the original samples are represented for comparison.

It is seen that the P/N ratios for both strains calculated from the values found in the three fractions agree well with those directly determined. In both cases there is a definite difference between the two strains of the order of magnitude of that found for the total material, which was found to be 5.77 mg phosphorus per g nitrogen (p. 352). As far as the fractions are concerned, it is seen that MT1 contributes only little to the total difference in P/N ratio, even though the difference in P/N ratio in this fraction was statistically highly significant. This is due to the fact, apparent from Table VII, that the total relative amount of this fraction is decreased in C57, even though this difference is not significant due to its large standard error. MT2 and MT3 contribute about equal amounts to the difference in P/N ratio. For MT2, this must be due to the qualitative difference in P/N ratio, since the mean contribution of this fraction to the total mitochondria is almost equal for the two strains. Finally, the contribution of MT3 to the strain difference must be due to the larger amount of material present in C57, since the qualitative difference in this fraction is not only non-significant, but in the opposite direction. Since the P/N ratio of MT3 is very high, a quantitative difference in this fraction will contribute relatively greatly to a difference in total P/N ratio.

Since the existence of a difference in the P/N ratio of the two strains appears to be well established, this total difference must be due either to a difference in one fraction alone or to several fractions. The evidence presented indicates that all three fractions contribute part of the higher P/N ratio of C57 mitochondria. The contribution of MT1 and MT2 seems to be solely due to a qualitative difference in P/N ratio. In MT3, on the other hand, there is no evidence for a qualitative difference in P/N ratio, and its contribution to the total difference in P/N ratio appears to be due entirely to the fact that it is increased in amount in C57 relative to the two other fractions.

The analysis indicates that the difference in P/N ratio between BALB and C57 mitochondria is a complex phenomenon in which at least two independent factors are involved.

DISCUSSION

The phosphorus/nitrogen ratios of mitochondria found in this study are lower than those described by Claude (7) for leucemic cells, but of the same order of magnitude as described by him for large granules from rat and guinea pig liver (6). As this author isolated mitochondria in electrolyte solutions, the preparations cannot be strictly compared, since it is known that microsomes are adsorbed to the mitochondria under these conditions. Muntwyler, Seifter and Harkness (17) as well as Paigen, Kaufmann and Wood (18) found P/N ratios for mitochondria isolated from rat livers of 78 mg P/g N, a value slightly lower than that obtained in the present experiment.

A large number of measurements of RNA/total N and of RNA/protein N ratios has been reported in the literature. Most of them (see review by Schneider and Hogeboom (26)) vary around a value of 11 mg RNA-P/g N. Only the values found by Barnum and Huseby (3), Huseby and Barnum (13) and Price, Miller and Miller (20, 21) are higher. The values for RNA/protein N ratio given in Table IV are even slightly higher than those found by Price, Miller and Miller (20, 21). These authors, in order to explain the difference of their values from those found by Schneider, Hogeboom and Ross (27) for mouse liver mitochondria, point to the following differences in technique: 1) possible difference in diet; 2) whether or not the livers were perfused; 3) whether or not the material was forced through a tissue mincer prior to homogenization; 4) concentration of the sucrose solution. Our technique agrees with that of Price, Miller and Miller as far as points 2 and 4 are concerned; but our material was not forced through a tissue mincer, excluding point 3 as a probable source of variation.

Schneider and Hogeboom (26) on the other hand suspect contamination with microsomal material as the possible cause of the difference. This is certainly true for our preparations. Schneider and Hogeboom (26) as well as Muntwyler, Seifter and Harkness (17) mention that they removed the uppermost fluffy layer of the mitochondrial sediment and added it accordingly to the microsomal fraction. Since it was found in our preparations that this fluffy material consisted microscopically of short rods clearly visible under the microscope, care was taken in the present experiments to keep this fluffy material in the mitochondrial fraction. Muntwyler, Seifter and Harkness (17) point out that this "fluffy layer" material is poor in nitrogen, but rich in RNA, and consequently in phosphorus. It could therefore well contribute to a higher P/N and RNA/N ratio. Laird, Nygaard, Ris and Barton (15) have isolated the material of this fluffy layer and proved that it consists of rods which resemble mitochondria in their enzymatic activity but microsomes in their RNA/protein ratio. The inclusion of the "small mitochondria" of Laird, Nygaard, Ris and Barton in the mitochondrial fraction seems sufficient to explain the high RNA/protein ratio obtained in the present experiments.

The main result of the present investigation may be summarized by stating that liver mitochondria from C57 Black males differ from those of BALB males by having a higher P/N ratio, and that this difference is at least in part due to a difference in RNA/protein ratio. Subdivision of our mitochondrial fraction has shown that this difference is due to at least two apparently independent components, a qualitative difference in the large mitochondria, and a quantitative difference in the small mitochondria. Since the small mitochondrial fraction MT3 may be presumed to contain the bulk of the "fluffy layer" mitochondria which according to Laird, Nygaard, Ris and Barton (15) are very rich in RNA, it must be concluded that a quantitative increase of this fraction in C57 will express itself in an increase in RNA in the total mitochondrial preparation. It is therefore not justified to conclude from our data that the qualitative difference found in MT1 and MT2 is also due to differential amounts of RNA though this possibility is not excluded.

Most authors assume that the particulate cytoplasmic material consists of two chemically and enzymatically well defined types of particles, mitochondria and microsomes. Chantrenne (5) and Paigen and Paigen (19), on the other hand, have suggested that the cytoplasmic particles consist of a continuous spectrum ranging uninterruptedly from the largest mitochondria to the small microsomes, and that the differences between different fractions are arbitrary and depending on the technique of isolation only. The data presented in the present paper agree with those of Chantrenne and of Paigen and Paigen by showing that the mitochondrial fraction can be subdivided into three subfractions with characteristic P/N ratios, the smaller particles having the higher P/N ratio. They have furthermore shown that the large particles and the small particles react in a differential way on the same genetic situation, the large ones by a qualitative difference between the strains, the small ones by a quantitative difference. This seems to bear out the finding of Laird, Nygaard, Ris and Barton (15) that the small mitochondria of the fluffy layer must be regarded as a separate qualitatively different type of particle. The properties of the intermediate fraction MT2 can be understood as those of a mixture of MT1 and MT3 material, with MT1 predominating. The data, therefore, do not contribute to the solution of this question beyond the point of providing independent evidence for a qualitative difference of the small mitochondria from the larger ones.

The strain difference in P/N ratio has turned out biochemically to be a complex character consisting of at least two different components. It is conceivable that genetically it will be complex too, particularly in view of the fact that the two strains investigated may be assumed to differ in a large number of genes. This would be in agreement with the well-known lability of the properties of mitochondria under the influence of environmental conditions and of the medium of isolation. It has been shown (4) that differences in genetic constitution lead to the establishment of different biochemical equilibria in the cell. It is therefore quite conceivable that these differences in intracellular composition would affect the qualities of the mitochondria.

It may, however, be possible that the relation between cytoplasmic particles and genetic constitution is a more direct one. It is well known that a large number of genes influence the enzymatic activities of the cell. Since it is well established that at least part of the enzymes are found localized in cytoplasmic particles, an influence of genes on the structure and function of cytoplasmic structures would be expected to result in an alteration of their enzymatic activities. An influence of genetic differences on the enzymatic activity of mitochondria is suggested by the observation of Lehninger and Kennedy ((16), p. 763 footnote) that the inhibition of fatty acid oxidase activity in preparations from rat liver by malonate is highly different in materials obtained from different strains. This enzyme complex has been shown to be concentrated in the mitochondrial fractions. Similarly, cases have been described in which individual genes control cytoplasmic particles with definite functions. It has been shown by Hanser (9) that the gene *wa* in the moth *Ephestia* controls the presence or absence of the precursor granules of pigment in the eye. Similarly, an influence of a group of individual genes on the chromoplast particles in the Malpighian tubules of the silkworm has been demonstrated (14). The possibility that individual genes may influence cytoplasmic particles more directly can, therefore, not be excluded. For the strain difference in liver mitochondria of the mouse, no conclusion can be drawn from the data presented on its relation to genes; it may be relatively direct or indirect, via the biochemical makeup of the cell, or a mixture of both possibilities.

SUMMARY

1. A significant difference in phosphorus/nitrogen ratio between isolated liver mitochondria from C57 Black and BALB/Ci males has been demonstrated.

2. In addition, variability in P/N ratio between experiments, probably due to experimental conditions, is apparent in the data. A slight effect of age in the younger animals is suggested, but not definitely proved.

3. The strain difference in mitochondrial P/N ratio is at least in part due to a difference in the relative amounts of ribosenucleic acids and proteins.

4. Results of experiments involving repeated washing of the mitochondrial preparations show that the P/N ratio remains constant after the first washing. This result makes it unlikely that the difference in P/N ratio between the strains is due to differential adsorption of microsomes.

5. The mitochondrial preparation was divided into three subfractions by differential centrifugation. The smaller subfractions, consisting of particles sedimented by higher centrifugal force, have a higher P/N ratio than the larger ones. Large mitochondria have a higher P/N ratio in C57 than in BALB. In small mitochondria, the P/N ratio in both strains seems to be the same, but more material is present in this fraction in C57 than in BALB. Since the "small mitochondria" fraction is rich in phosphorus, the larger amounts in this fraction contribute to the higher P/N ratio in C57.

6. It is concluded that at least two different types of particles are present in the mitochondrial preparations which differ in their reaction to the same genetic situation. The strain difference in mitochondrial constitution is a biochemically complex character consisting of at least two independent contributions.

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FURTHER INVESTIGATIONS ON THE CHANGE IN THE PIGMENT DURING EMBRYONIC DEVELOPMENT OF ECHINODERMS

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The finding that quantitative and qualitative changes occur in the pigments of the egg during embryonic development of *Paracentrotus lividus* (4) has led to the realization that the pigments may play some role in morphogenesis. It has thus seemed desirable to extend the investigation of pigment changes to other species in order to ascertain whether the phenomenon is of general occurrence.

The results presented here confirm that this is the case and indicate that it is in the gastrula stage that the most dramatic changes occur.

METHODS

Eggs of *Psammechinus microtuberculatum*, *Sphaerechinus granularis* and *Asterias glacialis* were used in these experiments. From each batch a measured sample of unfertilized eggs was collected. The remainder was fertilized and allowed to develop under continuous stirring. At different time intervals a measured quantity of the suspension was collected and transferred to a 10 cc glass-stoppered cylinder. After the eggs had settled the supernatant water was removed. 3 cc of ethanol + 7 cc of ethyl ether were added and the mixture was shaken vigorously. At the same time two samples of 0.1 cc were taken for determination of total nitrogen. Upon shaking the pigment passed into the ether. The extracts were collected and the extraction repeated until the eggs were colourless. The extract was washed with water, dried over Na_2SO_4 and brought to dryness in a stream of nitrogen. The residue was taken up in 4 cc of light petroleum ether (b.p. 35°–50°) and the spectrum of this *total extract* was read in the Beckman spectrophotometer between 320 mμ and 550 mμ. The value of the extinction at the point of maximum absorption was taken as a measure of the quantity of pigment.

The fractionation of the total extract was performed chromatographically as previously described.

RESULTS

Psammechinus microtuberculatum. The yellow colour of the gonads and unfertilized eggs of this species is caused by a mixture of β-carotene, echinenone and an unidentified epiphasic carotenoid with maxima (in light petroleum ether) at 400 mμ and 420 mμ. The relative percentages in the gonads are 18, 67 and 15 per cent, while in the eggs they are 11, 85 and 4 per cent. The point of maximum absorption of the total extract lies at 450 mμ for the unfertilized eggs and blastulae, at 455 mμ for gastrulae and at 330 mμ for plutei (Fig. 1). Fig. 2 shows the changes of the total quantity of pigment during early development. The quantity of pigment decreases rapidly during the first hours of development and attains a minimum at the morula stage. It then increases to the swimming blastula stage and a second decrease follows with a minimum in the gastrula stage. This is followed by an increase to the pluteus stage and there appears to be a decrease from that stage on. In the mid-gastrula stage a brick-red pigment appears; it is not extractable with ethyl ether but passes rapidly into acidified ethyl ether. From its properties this pigment appears to be a quinone.

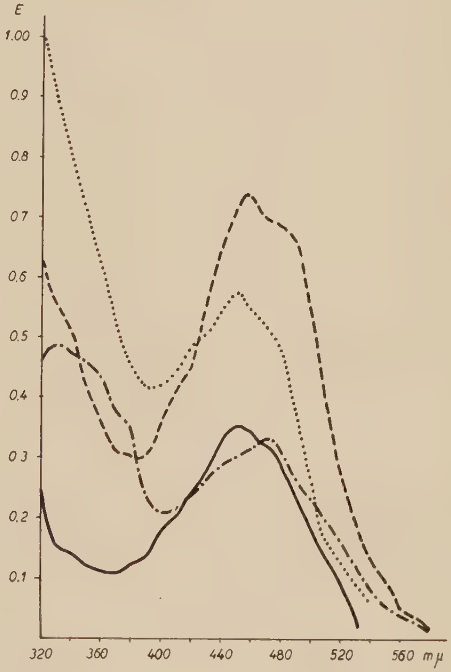

Fig. 1. Spectra in ethyl ether of the total extract of unfertilized and fertilized eggs of *Psammechinus*; ——— unfertilized eggs; ······· blastulae; – – – – – gastrulae; –·–·–· plutei.

Sphaerechinus granularis. As already described in detail (1) the distribution of carotenoids in the ovaries, testes and unfertilized eggs is quantitatively and qualitatively similar with only slight differences. Echinenone (70 per cent) and β-carotene and a related compound (16 per cent) are the main constituents. Dihydroxyxanthophylls exist in trace amounts.

In the period between fertilization and the gastrula stage the total quantity of pigment appears to undergo only a slight decrease. In the early gastrula, however, a rapid drop occurs (Fig. 2). At the same time the colour of the eggs turns from yellow to a reddish-pink. From this stage on the only carotenoid present is echinenone. At the pluteus stage the echinenone also disappears. After the extraction with ethanol-ethyl ether the eggs retain a red colour which is easily extracted in acidified ethyl ether. The absorption spectra of the total pigment in light petroleum ether at different stages is given in Fig. 3. Unfertilized eggs and blastulae have an absorption maximum at 450 mμ; gastrulae and plutei have two maxima at 325 mμ and 470 mμ.

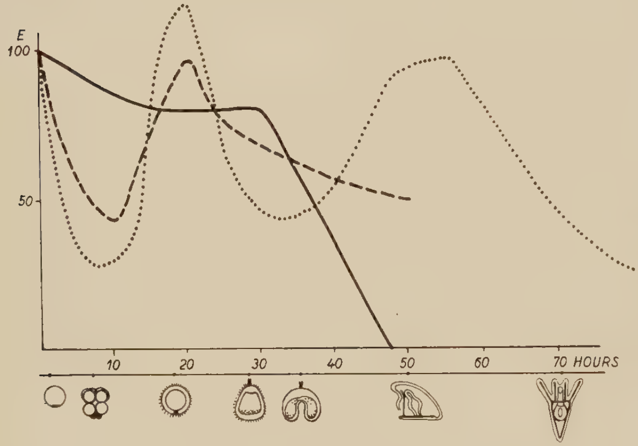

Fig. 2. Changes in the total content of carotenoids during development of *Psammechinus microtuberculatum* (·······), *Sphaerechinus granularis* (———), *Asterias glacialis* (– – – –). E_{max} of the extract of the pigment is referred to 10 mg N.

A preliminary separation of the components of the total extract has been carried out on columns of cellulose $CaCO_3$ or Al_2O_3 using different solvents and eluents. Five fractions have been separated in this way. Two of these fractions, however, seem to be inhomogeneous when isolated from extracts of gastrulae. The data are recorded in Table I.

TABLE I

Stage of development	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5
gastrulae	echinenone	—	inhomogeneous	inhomogeneous	—
plutei of 48 hours	—	present	present	present	echinocrome
plutei of 62 hours	—	present	present	present	echinocrome

Fraction 1 is echinenone and as already mentioned it is not found in stages later than the gastrula. Fraction 2 is an epiphasic carotenoid (phase partition, according to Karrer and Jucker (2)), between light petroleum ether and 90 per cent aqueous methanol (v/v), with absorption maxima at 330–405–475 mμ (light petroleum ether). It is present in plutei but does not occur in gastrulae. When fraction 3 is rechromatographed on cellulose or $CaCO_3$ using petroleum ether it can be eluted only with methanol and it gives a diffuse red band. In the phase partition it is hypophasic and it seems to be inhomogeneous in the gastrula stage. Fraction 4 is also hypophasic to 90 per cent (v/v) aqueous methanol. It is very strongly absorbed on cellulose and can be eluted only by acidified ethyl ether. It stays at the top of a cellulose column and can be taken up in ethyl ether only after the $CaCO_3$ has been dissolved in dilute HCl. It is insoluble in light petroleum ether. Its colour on the column is red but turns to orange in ethyl ether. Furthermore, it shows a characteristic colour change from red to orange when the pH of the medium is changed from acid to alkaline. This suggests its being a quinone. This fraction also seems to be inhomogeneous in extracts of gastrulae, while in the plutei it is clearly homogeneous (3). The absorption spectrum of fraction 5 in CS_2 proves it to be echinochrome. It is soluble in

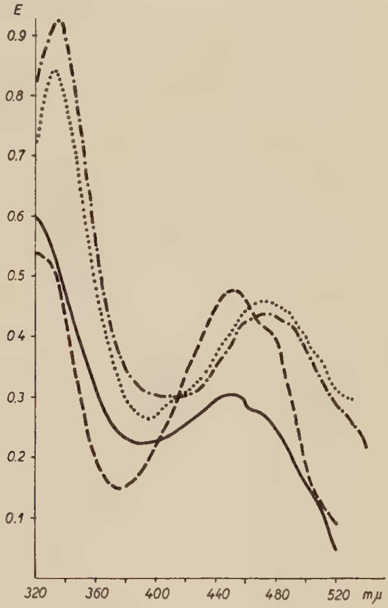

Fig. 3. Spectra in light petroleum ether of the total extract of unfertilized and fertilized eggs of *Sphaerechinus granularis*: – – – – – unfertilized eggs; ——— blastulae; · · · gastrulae; ·–·–·–·– plutei.

water made alkaline with bicarbonate and shows the typical colour change from violet to orange when the pH of the medium is varied. When the total extract in acidified ethyl ether is washed with water it passes quantitatively into the water phase and has a violet colour.

Asterias glacialis. The analyses of the carotenoid making up the red pigment of the eggs and ovaries of this species is under way. Only the changes in the total quantity of pigment in early stages of development are described here. The development has been followed only to the gastrula stage. The behaviour of the pigment of this species seems (Fig. 2) quite similar to that of *Psammechinus.* A strong decrease in the total quantity of pigment occurs to the morula stage. This is followed by an increase to a maximum in the swimming blastula stage and a second decrease to the gastrula stage. A new red pigment appears at the gastrula stage which in this case has also been found to be a quinone. The identification of this pigment is being attempted.

DISCUSSION

The data collected so far have some interesting implications. The variations in the total quantity of pigment during early embryonic development of *Psammechinus* and *Asterias* suggest that carotenoids synthesis may occur. These embryos do not feed until the pluteus stage. Thus, if carotenoid synthesis did occur, it would be the first case described for the animal kingdom in which carotenoids were synthesized from a non-carotenoid precursor.

The pigment variations during embryonic development of different species of echinoderms (*Psammechinus, Asterias, Sphaerechinus* and *Paracentrotus*) suggest that the carotenoids may play an active part in morphogenesis. However, it will not be possible to understand the full significance of these findings until the metabolic pathways of the pigments are known. While β-carotene and echinenone are found in all the species so far examined the xanthophylls are not. This fact, taken together with the fact that the curves of the variations in total quantity of pigments during development are different may suggest different metabolic pathways.

In spite of these differences, however, there are two similarities between all species thus far examined, *i.e.* the greater or lesser variations in the total quantity of pigment and the appearance of a new type of pigment, probably a quinone, in the late gastrula stage of the species examined. *Sphaerechinus* is unique in that there is a complete disappearance of carotenoids by the pluteus stage.

SUMMARY

In the eggs of *Psammechinus microtuberculatum, Sphaerechinus granularis* and *Asterias glacialis* the total quantity of pigment appears to vary during early development.

In *Psammechinus* cyclic changes occur with maxima in the swimming blastula and pluteus and minima at the end of cleavage and in the gastrula. Similar changes are observed in *Asterias.*

In *Sphaerechinus,* the total quantity of pigment undergoes only a slight decrease during cleavage and then remains constant until gastrulation, when a sudden drop occurs.

In the eggs of *Psammechinus,* β-carotene, echinenone and a third unidentified carotenoid are found. *Sphaerechinus* eggs contain β-carotene, echinenone, a related compound and dihydroxanthophylls. The analysis of the carotenoids of unfertilized eggs and gonads of *Sphaerechinus* has already been described.

A new pigment, probably a quinone, always appears in the late gastrula. It has been submitted to a preliminary analysis in the case of *Sphaerechinus.*

The author wishes to express her indebtedness to Professor A. Monroy for his suggestions, advice and criticisms in the preparation of the manuscript and to Dr. P. Dohrn for his help in culturing the stages of *Asterias.*

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CONTINUOUS OBSERVATION OF THE RESPIRATION OF SKIN *IN VITRO*

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As part of a fundamental study of the ways in which skin is damaged by various toxic substances and radiations it was desired to culture skin slices *in vitro* and to follow their respiration over a period of several days. Medawar (6) had shown that skin slices may be maintained floating on the surface of a fluid medium, and that during this period epidermal cells migrate along the cut surface of the dermis. A modification of this method has been used previously by the author to study the toxicity of antibiotics to skin (2). In order to provide further information upon the toxicity of various agents, a differential capillary respirometer has been devised which will permit continuous observations of oxygen uptake for indefinite periods and the admission of any gas mixture or liquid to the tissues during the experiment. The apparatus may be operated in a standard 37° C water bath and does not require very fine thermostasis. The essential improvement upon models previously described is the design of a simple tap which permits simultaneous opening and closing of the two chambers. This eliminates the need for a paraffin wax seal of the capillary as used by Cunningham and Kirk (3) or for needle valves as described by Kirk (5). Neither of these devices will function in a water bath at 37° C.

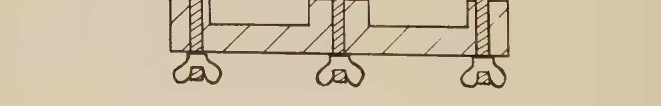

Fig. 1. Sectional diagram of respirometer.

CONSTRUCTION

The respirometer is made from two brass plates 8.5 × 5 × 1.25 cm. In each plate are turned two symmetrically placed chambers of 2.5 cm diameter. In one plate (to become the lower part of the respirometer) the chambers are turned to a depth of 6 mm and in the other the chambers are made 4 mm deep. The opposing surfaces of the plates are ground flat on a glass sheet (used photographic plate) with fine carborundum. When placed together the chambers in each part should correspond exactly. The two halves are held together by five symmetrically placed brass bolts (4 BA) with wing nuts, the surfaces being lubricated by a very thin film of silicone grease.[1]

In the roof of each chamber two holes are drilled. One, 2 mm in diameter, will provide access for gases and liquid and into the other the capillary is sealed with Araldite.[2]

The capillary is bent to the shape shown in the diagram—the straight portion being about 13 cm in length. It has been found that soft glass capillaries of bore 0.4–0.5 mm are most generally useful, but clearly the capillary size may be adjusted to the needs of the problem.

As indicator droplet fluid, specially prepared kerosene as recommended by Glick (4) has been found satisfactory. The droplet is introduced into the capillary with a fine hypodermic needle. A plastic scale graduated in 0.5 mm is attached to the capillary.

The gassing tap is made from a piece of Perspex 40 × 20 × 6 mm and is rounded at the ends. Three holes are drilled in this. Two holes symmetrically placed will fit exactly over the holes in the roof of the chambers while the third, centrally placed, admits the central bolt of the respirometer about which the tap swivels. The opposing surfaces are lubricated with silicone grease. A blood transfusion needle or some tube of similar dimensions is sealed with Araldite into each of the outside holes of the gassing tap. The ends of these tubes will project from the surface of the water bath.

OPERATION

Skin slices are cut free-hand from guinea-pig ear. These can be cut approximately 0.2 mm thick and with practice very even. Only thin slices are used; thicker slices showed considerably less oxygen consumption per mg. The skin slices are weighed on the 10 mg torsion balance and floated by surface tension on the surface of the culture medium in the reaction chamber. The medium consists of:

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[1] Dow-Corning Stopcock Grease obtainable from W. Edwards & Co. (London) Ltd., Manor Royal, Crawley, Sussex.

[2] Araldite Type 101 is a synthetic resin manufactured by Aero Research Ltd., Duxford, Cambridge.

0.5 ml serum (usually homologous), 0.3 ml Krebs-Ringer phosphate, 0.1 ml glucose 5 per cent, 0.1 ml streptomycin 400 μ/ml.

1 ml of this medium is placed in a glass dish 6 mm deep and approximately 2 cm internal diameter in each of the chambers. These dishes rest on circles of filter paper which cover the bottom of the chambers and are soaked with 0.2 ml of 2 per cent KOH.

The apparatus is bolted together with the gassing tap in the open position. The respirometer is placed on a rack in the water bath with the ends of the gassing tubes projecting above the water. The chambers are then gassed if so desired by passing a stream of oxygen down a fine, long hypodermic needle introduced into the chamber via the gassing tube; the indicator droplet is adjusted to the appropriate position, and the gassing tap closed. Readings are commenced after 30 minutes has been allowed for thermal equilibration.

When the indicator droplet has reached the end of the capillary the gassing tap is opened and by gentle pressure with the finger on the end of the gassing tube it may be returned to its original position.

Formulae for the use of the differential respirometers are given by Glick (4). It has been found that the simple formula $\Delta \frac{V}{p} = 2\,ad$ where $\Delta \frac{V}{p} =$ volume change, a = cross section of capillary and d = movement of droplet, is sufficiently accurate for our present purposes. This formula holds good when the chambers are of equal size and the gas volume change is small in relation to chamber size.

EXPERIMENTAL

The respirometer has been subjected to certain tests to define its accuracy and working limits. Since it was designed for use with skin cultures, skin has been used as the biological material, and as histological examination was required, the figures for oxygen uptake given below are based upon initial wet weights. The mean wet weight/dry weight ratio of the skin slices was 3.5/1.

Table I shows the oxygen uptake of different weights of skin taken from the same animal.

The experiment was performed by commencing observations with 21.9 mg of skin in the reaction chamber. By cutting the slices to recognisable shapes it was possible to abstract known weights at intervals of 1 $^1/_2$ hours. It will be noted that a constant value for the oxygen uptake is obtained for weights between 20 and 5 mg. Any failure of gas diffusion or carbon dioxide absorp-

TABLE I

The oxygen uptake of varying weights of skin.

Weight of skin	μ l.O$_2$/mg/hr.[1]	
	Mean	S.E. of mean
21.19 mg	1.04	0.005
16.52 mg	1.06	0.008
9.74 mg	1.06	0.008
4.44 mg	1.04	0.01

TABLE II

Effect of depth of medium on oxygen uptake.

Depth of medium	Slice No. 1 9.55 mg		Slice No. 2 11.82 mg		Slice No. 3 7.56 mg		Slice No. 4 10.06 mg	
	μ l.O$_2$/mg /hr.[2]	S.E. of mean	μ l.O$_2$/mg /hr.	S.E. of mean	μ l.O$_2$/mg /hr.	S.E. of mean	μ l.O$_2$/mg /hr.	S.E. of mean
1.5 mm . .	1.05	.012	1.13	.009	0.94	.002	0.91	.006
4.5 mm . .	1.07	.005	1.16	.005	0.96	.007	0.92	.007

TABLE III

Effect of submerging skin slices.

	Slice No. 1. 7.64 mg		Slice No. 2, 14.64 mg	
	μ l.O$_2$/mg/hr.[3]	S.E. of mean	μ l.O$_2$/mg/hr.	S.E. of mean
Floating	1.13	.007	1.13	.004
Submerged 1.5 mm	0.88	.006	0.65	.006
4.5 mm	0.69	.008	0.50	.008

tion would have caused lower values to be obtained with the larger amounts of tissue.

Table II shows the effect of varying the amount of medium upon the respiration of the same skin slice.

This shows that the depth of medium does not affect the respiration values obtained and indicates that no stirring is required. That this finding is dependent upon the skin floating is shown by Table III in which the effect of varying depth of medium is shown upon submerged skin slices. There is a reduction in the oxygen consumption as the depth of medium is increased. The extent to which the observed values dropped was variable as might be expected. The reduction is presumably due to the failure of oxygen to reach the tissue because of its low solubility and slow diffusion rate.

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[1] Based on 6 observations at 15-minute intervals.
[2] Based on 8 observations at 15 minute intervals.
[3] Based on 6 observations at 15 minute intervals.

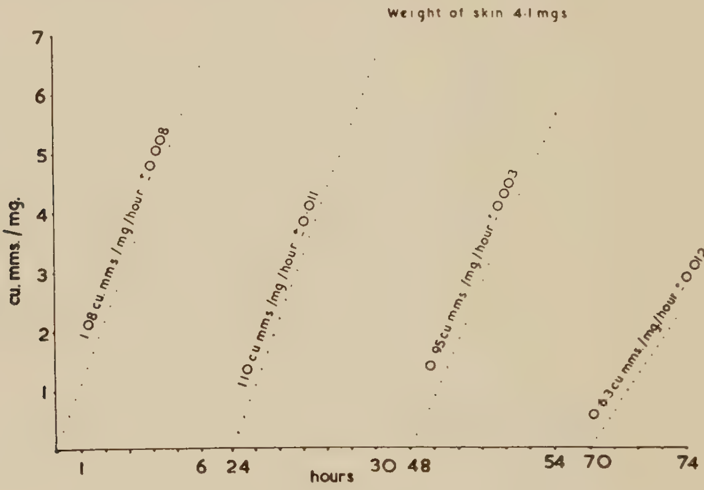

Chart 1. Oxygen uptake of skin slice over 3 days.

The experiments show that constant respiratory rates may be obtained for skin slices for periods of up to 6 hours, and that the mean error of a respiratory rate based on 6 or 8 readings at 15 minute intervals is 0.008 µl/mg/hr. or 0.8 per cent of the observed rates.

Observations of the oxygen uptake of skin slices for periods of more than 6 hours are liable to be affected more by the biological state of the tissue than by intrinsic errors of observation. For example, the cycle of mitotic activity in skin has been shown to be associated with variations in oxygen uptake (1). Moreover, when skin is cultivated in a fluid medium migration of the epidermal cells along the cut surface of the dermis occurs (6). It is not known whence the energy is derived for this process.

Preliminary experiments have shown that for periods of more than 6 hours the course of the oxygen consumption is affected by the weight of tissue used. If weights of 10–20 mg are cultivated in 1 ml of medium there is a period of 6–8 hours during which the oxygen uptake remains steady. Thereafter a gradual drop in the QO_2 values occurs and these approximate to zero by 48 hours. Histological examination of the skin at this stage shows that it has become necrotic. The failure of the larger amounts of tissue to survive is likely to be due either to the exhaustion of some serum constituent or to the accumulation of some toxic metabolite. Observations of the pH (see below) show that accumulation of lactic acid is not a valid explanation.

Using skin weights of 5 mg the respiratory rate remains relatively constant for 48 hours; frequently there occurs a period of some 6 hours between the 12th and 24th hour during which the QO_2 values are raised some 10 per cent. As this period occurs during the night, only a few observations of this kind have been made so far. After about 48 hours a rapid reduction of oxygen consumption occurs and by 72 hours the values obtained are about 60 per cent of the original rate. A typical example is shown in Chart 1. At this stage the skin is histologically normal. Moreover migration of the epidermal cells across the cut surface of the dermis has occurred. Examination of the skin at various intervals after incubation shows that this migration never occurs before 24 hours; early migrating cells at the shoulders of the culture may be seen at 48 hours but the process is never completed before 72 hours. Thus migration takes place during the period of reduced total oxygen consumption of the skin.

Whether the reduced oxygen consumption represents a change in the metabolism of the cells associated with migration or is due to other factors cannot fully be discussed in this communication. It might be explained on a simple mechanical basis in that the covering of the dermis by epidermal cells precludes the adequate oxygenation of the culture. This seems to be the most likely explanation as small patches of necrosis are observed in the dermis if the culture is continued for 5 days.

Finally observations have been made of the pH of the medium. The fresh medium has a pH of 7.8–8.1 and within 1 hour this has risen to 8.5 in both culture and control chambers. Thus at the time when readings are commenced the pH of the culture medium is 8.5. After 24 hours the values for the pH of the culture medium are between 8.0 and 8.5—depending upon the amount of tissue present and at 72 hours are between 7.0 and 7.5. During this period the pH of the medium in the control chamber lies between 8.5 and 9.0. The production of lactic acid by the skin may reasonably be supposed to counteract the excessive rise of pH caused by removal of O_2. It would seem also that medium having a pH of 8.0–8.5 has no deleterious effect upon skin. Similar observations have been made by Trowell (7) in respect of lymphocytes.

SUMMARY

1. A differential microrespirometer has been devised which will permit continuous observation of the respiration of skin slices for periods up to 3 days.

2. Gas mixtures and liquids can be added to cultures during the experiment.

3. For periods of observation of up to 6 hours skin slices of 10–20 mg initial wet weight may be used; for periods up to 3 days skin slices of 5 mg may be used.

4. For tissue weights of this order the mean error of an observation based on 6 or 8 readings at 15 minute intervals is less than 0.01 μ l/hour or approximately 1 per cent of the observed rates.

I wish to thank Mr. J. R. Cooper, F.I.M.L.T., Technical Research Assistant, for his invaluable help at all stages of this work. I also acknowledge with pleasure much useful advice and assistance given to me by Dr. O. A. Trowell of the M.R.C. Radiobiological Research Unit, Harwell.

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STUDIES ON MINERALIZED DENTAL TISSUES

I. A MICRORADIOGRAPHIC AND AUTORADIOGRAPHIC INVESTIGATION OF TEETH AND TOOTH GERMS OF NORMAL DOGS

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Probably the mechanism of mineralization is essentially the same in all hard tissues of the body. When working on problems concerning mineralization of dental hard tissues one should also pay attention to the evidence emerging from investigations of bone tissue.

In recent years much fresh evidence concerning the mineralized tissues has been secured by means of biophysical methods. The application of tracer techniques to this field has thrown new light on the chemical and physical properties of the inorganic constituents of the hard tissues. Mostly P^{32} has been used as tracer element, but recently the uptake of Ca^{45} and other isotopes has also been studied.

Already the very first experiments with P^{32} (9) disclosed a rapid uptake of the isotope by the bones and teeth of rats. This uptake was shown to be most marked in areas of rapid growth. The distribution of P^{32} in tooth tissues was subsequently studied by several investigators (6, 7, 15, 20). It has thus been shown that the labelling with P^{32} is most pronounced at certain sites of the dental hard tissues and that the uptake is extremely rapid in areas undergoing mineralization (4, 5, 17, 18).

Experiments on tooth tissues carried out with Ca^{45} suggest that this isotope behaves similarly to P^{32} with respect to the mineralized tissues and essentially the same order of uptake is found for both P^{32} and Ca^{45} (3, 8, 14).

As has recently been demonstrated S^{35}-labelled sulphate enters bone tissue in similar structures as do Ca^{45} and P^{32}. However, a proportion of the S^{35} labelling of bone tissue affects the organic constituents of the mineralized tissue (10).

With the aid of autoradiography the uptake of radioactive isotopes can be localized in certain areas of the mineralized tissue provided the resolution is sufficiently high. The degree of mineralization in these structures can, however, not be estimated by means of ordinary histological methods. Attempts have been made at demonstrating the variations in mineralization of teeth by means of radiographic procedure with soft X-rays (1, 2). In these early investigations rather thick sections were used and the resolving power of the photographic emulsion was not very high; hence the finest structures and gradients of mineralization escaped observation. With a microradiographic method described by Engström and Wegstedt (13) it is now possible to obtain accurate information as to the content of mineral salts in the hard tissues. Using this microradiographic technique combined with autoradiography it has recently been shown that the uptake of P^{32} in bone tissue takes place in certain areas *viz.* areas with low content of mineral salts. These structures represent newly formed bone tissue (11).

The aim of the present investigation is to study the formation of mineralized tissues in the teeth of normal dogs with the aid of a combined microradiographic and autoradiographic technique. In the autoradiographic procedure both Ca^{45} and S^{35} are used in order to obtain information as to both the inorganic and organic constituents of the tooth tissues.

MATERIALS AND METHODS

In the present investigation two litter-mate dogs fed an adequate diet were used. At the age of 3½ months one of them was injected intravenously with 10 mC carrier-free $Na_2S^{35}O_4$ and the other with 1.5 mC carrier-free $Ca^{45}Cl_2$. Four days after the injection of the isotopes the dogs were killed; the mandibles were removed, cleared of soft tissues and afterwards fixed with alcohol. To secure exact information as to the positions of the separate tooth germs roentgenograms of the mandibles were taken. With a rotating saw thin sections were then prepared from selected areas of the mandibles. Some of the specimens which consisted of the tooth germ only, were immediately ground on glass plates to a thickness of 50–100 μ. Large specimens containing both the deciduous tooth, mandible and tooth germ were first embedded in methylmetacrylate and afterwards ground to a thickness of 80–200 μ.

The ground specimens were microradiographed with the aid of, in principle, the procedure described by Engström and Wegstedt (13). A Philips diffraction unit with copper Kα-radiation filtered Ni served as radiation source. The tube was operated with 30 kV and 12 mA. The focus to film distance was 25 cm. The microradiographs were recorded on Eastman Kodak Spectroscopic Plate No. 649. This emulsion is extremely fine-grained and has a resolving power of about 1 μ. The microradiographs were enlarged by microphotography on Kodak 0250 Plates.

After having been microradiographed the specimens were used for autoradiography. They were sandwiched between sheets of Agfa Printon Film and placed in a special holder to ensure a good film-specimen contact. The film was exposed for 1 to 3 months. Some of the specimens were afterwards mounted in xylene, and microphotographs were taken in transmitted light.

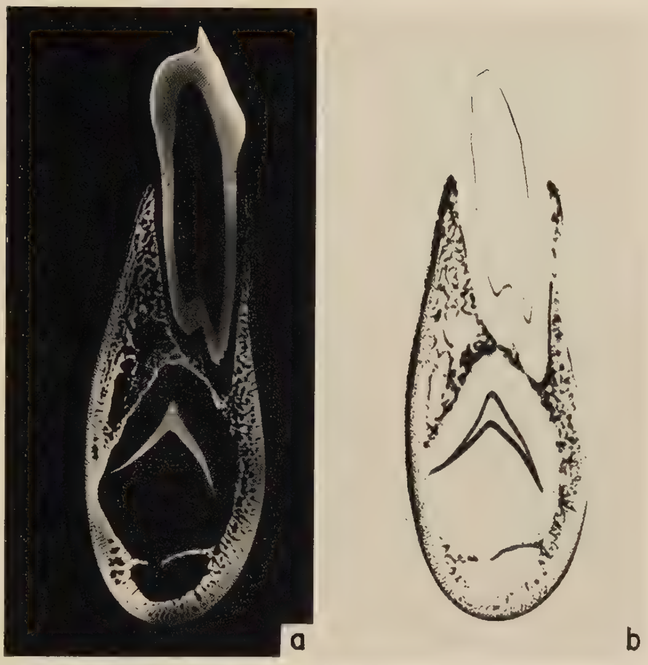


Fig. 1. Ca^{45}-labelled dog. Microdiograph (a) and autoradiograph (b) of an 80 μ thick longitudinal ground section through the lower jaw. Note high uptake of isotope along the inner periosteal surface of the jaw. The labelling of the deciduous tooth is restricted to the pulpo-dentinal border and to the cementum (the uptake in the latter tissue not visible in the picture). The tooth germ shows a marked uptake along the pulpo-dentinal border and the surface of the enamel. × 3.5.

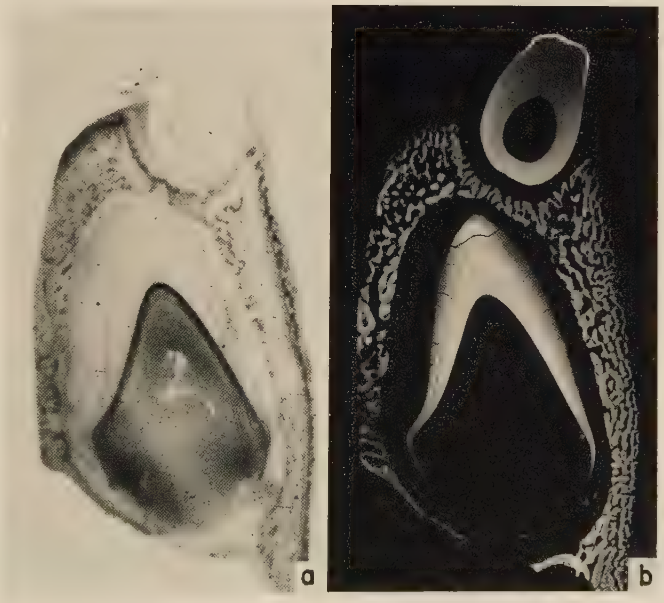


Fig. 2. S^{35}-labelled dog. Autoradiograph (a) and microradiograph (b) of an 80 μ thick longitudinal ground section through the lower jaw. The most striking differences versus Fig. 1 are the pronounced labelling of the pulp tissue of the tooth germ, and a faint uptake along the dentinoenamel junction. × 6.3.

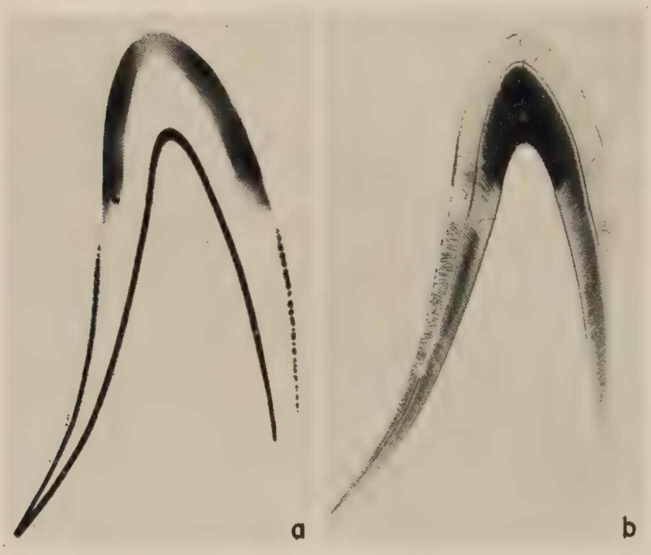


Fig. 3. Ca^{45}-labelled dog. Autoradiograph (a) and transmitted light picture of a $70\,\mu$ thick longitudinal ground section through the developing lower permanent canine (b). Diffuse labelling of the enamel throughout its whole breadth at the tip of the tooth. The uptake in the rest of the tooth is localized at the pulpodentinal border and along the enamel surface. (Part of the enamel was lost after the preparation of the autoradiograph.) × 5.

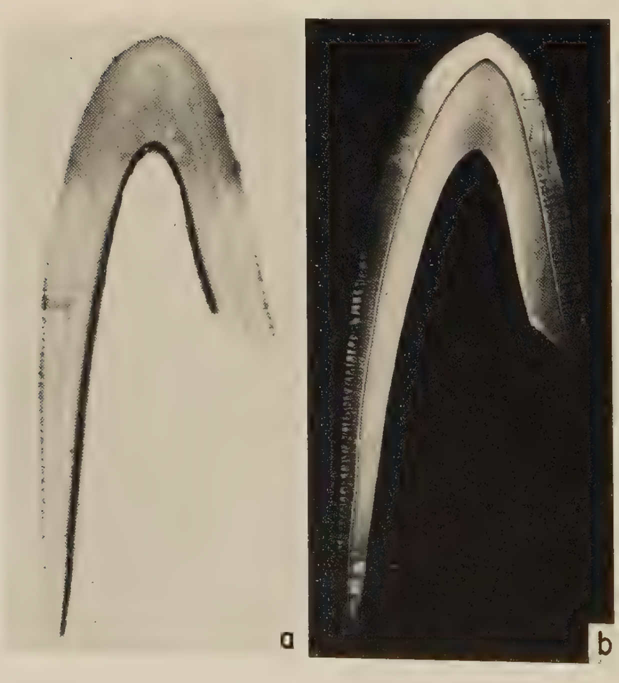


Fig. 4. S^{35}-labelled dog. Autoradiograph (a) and microradiograph (b) from a $70\,\mu$ thick longitudinal ground section through the developing lower permanent canine. Marked uptake of isotope along the pulpodentinal border, and faint labelling along the dentino-enamel junction and the enamel surface. Diffuse labelling throughout the whole enamel at the tip corresponding to an area with high X-ray absorption as seen in the microradiograph. × 6.5.

Fig. 5 (right). Ca^{45}-labelled dog. Autoradiograph (a) and 100 μ thick longitudinal ground section through the lower jaw (b). Uptake of isotope in a similar manner as in Fig. 1, except in the tip of the tooth germ where the enamel is diffusely labelled throughout its breadth. × 3.5.

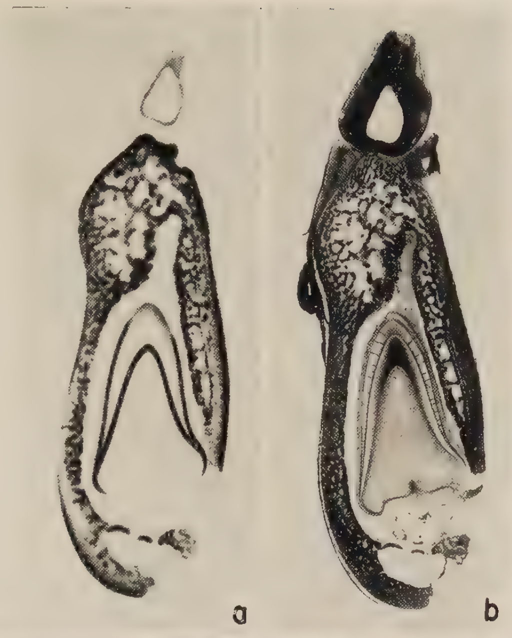
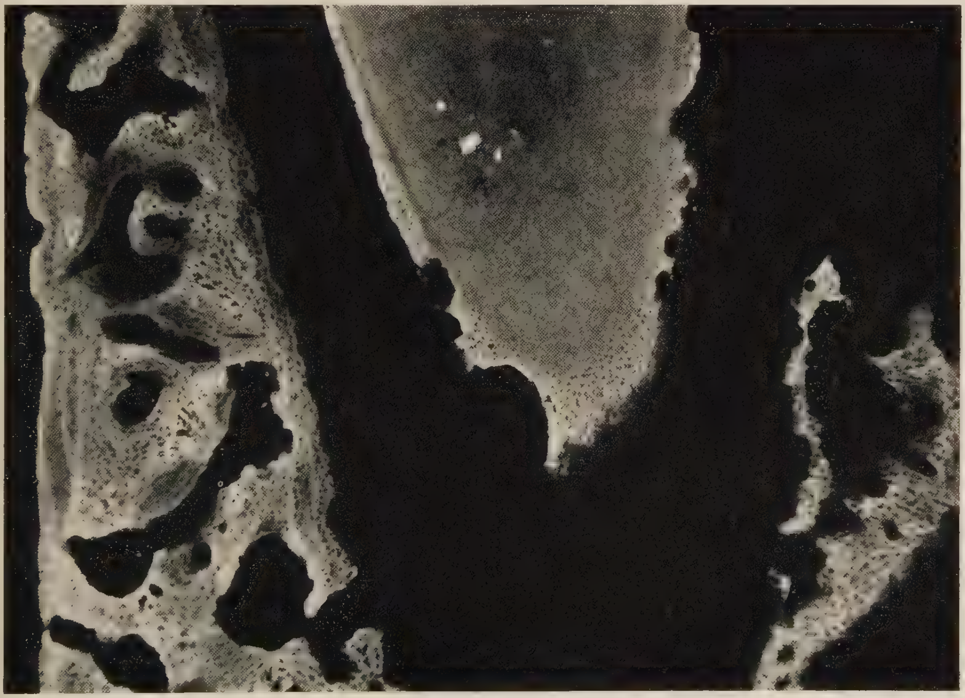


Fig. 6 (below). Microradiograph showing the root tip of a deciduous tooth with surrounding bone tissue. Resorption cavities in the cementum. × 60.



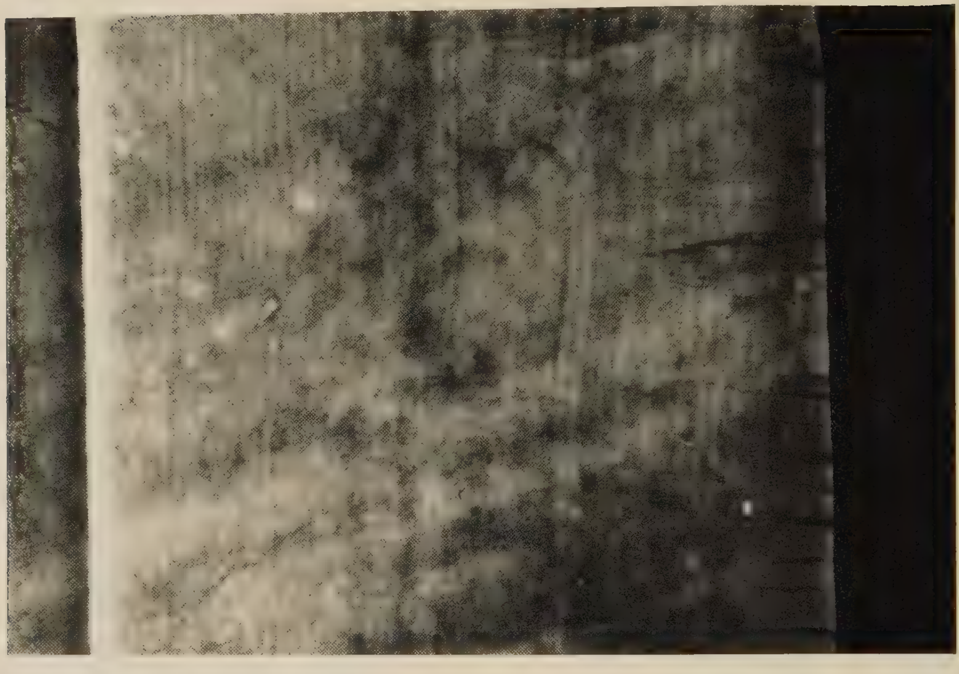


Fig. 7. Microradiograph showing incremental lines in the enamel of a tooth germ. Note highly mineralized enamel at the dentino-enamel junction. × 165.

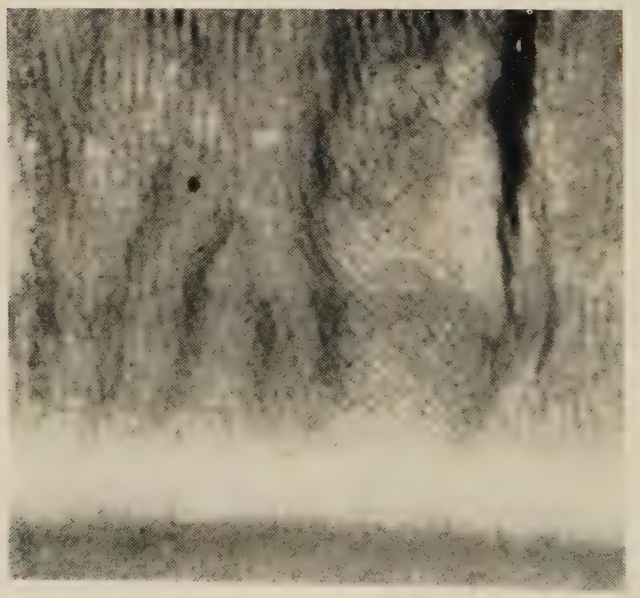


Fig. 8. Microradiograph showing enamel prisms arranged in bundles at the dentino-enamel junction of a tooth germ. × 270.

RESULTS

The autoradiographs from specimens of the lower jaw of the dog labelled *in vivo* with Ca^{45} (Fig. 1) reveal a high uptake of the isotope in certain structures of the bone. It could be shown that this uptake is localized in the periosteal areas and especially on the inner surface of the jaw. There is, however, also a considerable uptake throughout the bone tissue in those structures where the microradiographs show a low degree of mineralization. In the deciduous teeth the autoradiographs indicate a definite labelling of the tissue in a narrow zone corresponding to the pulpo-dentinal border, and there is also a faint labelling of the cementum. The tooth germs of the lower jaw show a marked uptake within a sharply limited zone along the pulpo-dentinal border, and a somewhat narrower zone of uptake along the surface of the enamel. In some of the autoradiographs (Fig. 1) this outer zone of uptake has the same width along the whole enamel, but in others (Figs. 3 and 5) there is a fainter uptake in the cusps throughout the whole depth of the enamel. This latter type of labelling of the enamel affects areas of varying size in different tooth germs, cp. Fig. 3 and Fig. 5.

Specimens from the lower jaw of the dog labelled with S^{35} give autoradiographs indicating uptake of isotope in certain areas of the tissue (Figs. 2 and 4), which correspond to those showing uptake in the Ca^{45}-labelled specimens. Hence, as in the case of Ca^{45}, there is a high uptake of S^{35} in the periosteal areas, and also in certain structures of the bone; moreover in the deciduous teeth, there is a narrow labelled zone of the pulpo-dentinal junction. In the tooth germs the pulpo-dentinal border reveals a high uptake, and the outer surface of the enamel also shows a well defined fine line indicating uptake of S^{35}; furthermore there is an indication of a diffuse labelling throughout the whole enamel at the cusps of certain tooth germs. However, the autoradiographs obtained with S^{35} disclose several features which definitely deviate from those obtained with Ca^{45}. There is thus also an uptake in the soft tissue, and this is well demonstrated in the pulps of the tooth germs (Fig. 2). This labelling is diffuse all over the pulp, but it becomes more pronounced in a rather broad zone towards the dentin where the uptake is very high in a narrow band corresponding to the pulpo-dentinal border. Careful examination of the uptake located here also discloses that this band consists of two equally dark lines quite close to one another. The autoradiographs also show a thin line of uptake corresponding to the dentino-enamel junction, and this line is not found in the autoradiographs obtained with Ca^{45}.

The microradiographs of ground specimens from the lower jaw show a bone tissue in rapid rebuilding with plenty of irregular resorption cavities often lined with bone tissue of low mineralization. Haversian systems with different contents of mineral salts are also seen. In the deciduous teeth the enamel is highly mineralized, and the dentin has a considerably lower content of minerals. The cellular cementum at the root tip (Fig. 1) displays several resorption cavities and is more markedly mineralized than the adjacent dentin. In the tooth germs the dentin is rather highly and uniformly mineralized (Figs. 2 and 4). The boundary to the pulp, however, shows a low content of minerals in a narrow zone which has a wavy appearance. Along the dentino-enamel junction there is an area which is less mineralized, and where the content of mineral salts drops towards the enamel. The enamel shows a thin zone of very high mineralization in its innermost part, and towards its surface the content of minerals is grossly uniform. On the other hand, in the cusps there is sometimes an indication of higher mineralization throughout the whole width of the enamel (Fig. 4). Along the outer surface of the enamel occasionally a thin line is noted with low absorption, separated from the mineralized enamel by a fine, non-absorbing zone. At high magnification (Figs. 7 and 8) the microradiographs disclose an abundance of thin, parallel lines with higher X-ray absorption, separated from each other by bands with lower absorption. These lines run at a low angle to the dentino-enamel junction. There are also thin bands with high absorption separated by small spaces of lower absorption running grossly perpendicular from the dentino-enamel junction to the surface of the enamel (Fig. 8). These bands are often arranged in bundles which are running in somewhat different directions. The degree of irregularity of these bundles varies in different parts of the enamel.

DISCUSSION

The uptake of radioactive isotopes in the salts of mineralized tissues can be interpreted in several ways. This uptake may be the manifestation of ionic exchange reactions between the minerals and the tissue fluids (19), but it may also indicate incorporation in newly deposited mineral salts (16). It has been shown that ionic exchange reactions occur to a greater extent in young bone than in old (11), and it is probable that this observation holds true for all mineralized tissues. In areas where deposition of minerals is going on ionic exchange reactions may thus play a role; however, it seems probable that in the material we have used most of the *in vivo* uptake results from deposition of mineral salts.

With the autoradiographic procedure used in this investigation no quantitative data can be obtained concerning the uptake of isotopes. The autoradiographs merely show what structures are being labelled, but in a rough way great differences in uptake of different structures in the same autoradiographs might be interpreted as indicating different degrees of uptake.

The discussion of the results of the autoradiographic procedure with Ca^{45} can be restricted to the mineral constituent alone, since it has been shown that the matrix does not show any appreciable uptake of Ca^{45} (12). As to S^{35}, however, the matrix must also be taken into account, as it has been demonstrated that in bone tissue both the mineral constituent and the matrix are being labelled (10).

It has been demonstrated that the *in vivo* uptake of Ca^{45} occurs in certain structures. These areas have a low degree of mineralization and are newly formed (10). The localization of Ca^{45} in certain structures of bone tissue has been shown to be the same as that which has been demonstrated for P^{32} (12). The high uptake of Ca^{45} in the dentino-pulpal border of the tooth germs indicates that deposition of mineral salts is proceeding at this site. Precise localization of this uptake to a certain structure of the microradiograph is not possible. However, it seems to be located in the predentin zone, but it might also affect some smaller parts of the adjacent structures. Also as regards the zone of labelling at the surface of the enamel, it is even here not possible to state with absolute certainty whether this labelling is situated inside the evenly mineralized part of the enamel, although this seems to be most probable. It might, however, also be located at the outer border of the enamel, *viz.* in the region which has a low content of mineral salts and is separated from the "highly" mineralized enamel by a thin zone without appreciable X-ray absorption. The uptake throughout the enamel in the cusps of certain tooth germs is probably to be interpreted as the beginning of a new mineralization phase up to high levels. This process seems to have advanced to different degrees in different tooth germs, possibly depending on the degree of maturity of the tissue. The microradiographs also seem to disclose a somewhat higher X-ray absorption in these parts of the tooth germs (Fig. 4). This interpretation is in close agreement with the earlier hypothesis concerning secondary mineralization of enamel (2).

The labelling of bone tissue with S^{35} has been shown to have the same general pattern as that obtained with P^{32} and Ca^{45}. However, there is the above mentioned difference as to the localization in the tissue, sulphate being incorporated both in the organic and inorganic constituents of bone tissue. As regards the tooth germs, the labelling with S^{35} occurs in the areas also labelled with Ca^{45} (the pulpo-dentinal border and the surface of the enamel) but it is also present at other sites. The uptake of S^{35}, which has the same general shape and occurs in the same areas as Ca^{45}, might be attributed to an incorporation of sulphate in the newly deposited mineral salts.

The uptake of S^{35} at the enamel surface could possibly be considered as due to incorporation in the matrix of enamel. This matrix is said to have the characteristics of keratins, and the keratins contain SH-groups. However, as S^{35} was given as sulphate, and sulphate does not participate in the formation of SH-groups, the possibility of producing a labelling of keratins by administration of $Na_2S^{35}O_4$ can be ruled out; hence it seems more reasonable to suppose that S^{35} is deposited at this site together with the minerals.

The labelling occurring in the other areas must be interpreted in another way, however. The uptake localized in the pulpo-dentinal region does not show the same general pattern as that obtained with Ca^{45}. In the latter instance, there is one even line of uptake running along the whole border. In the autoradiographs obtained with S^{35} we have found a diffuse labelling of the pulpal soft tissue, but at the dentinal border there are two closely adjacent lines of uptake. The uptake of S^{35} in this area also seems to be much more pronounced than the uptake at the surface of the enamel, an observation which also is opposed to what has been observed in the Ca^{45}-labelled specimens. These findings seem to indicate that the uptake of S^{35} at the pulpo-dentinal border is dependent upon an incorporation both in the organic and inorganic constituents of the tissue. In this area a brisk formation of matrix is going on, and this matrix comprises, *inter alia*, mucopolysaccharides containing sulfuric acid; it seems thus probable that the sulphate is incorporated in this fraction. When the minerals then are deposited in the matrix, sulphate is also incorporated in the crystal lattice. The uptake of S^{35} at the dentino-enamel junction does not correspond to any similar uptake of Ca^{45}. Consequently it does not seem probable that it has anything to do with the inorganic constituents of the tissue but rather with the organic ones. The mechanism of this deposit in the tissue is, however, obscure.

Apart from the results obtained by correlating the autoradiographs with the corresponding microradiographs, a more detailed examination of the latter revealed some interesting features. Thus, the thin lines of varying X-ray absorption running at low angles with the dentino-enamel junction correspond to the incremental lines of growth (Fig. 7). These lines are also seen in the ground sections under the microscope in transmitted light.

At high magnification the enamel-prisms could also be seen in the microradiographs, as the interprismatic substance has a lower X-ray absorption

(Fig. 8). The prisms are seen to be arranged in bundles which run irregularly towards the surface of the enamel.

Another interesting feature disclosed by the microradiographs is noted in Fig. 6. The root tip of the deciduous tooth exhibits resorption cavities in the cementum (cf. Fig. 1 a), and a bone trabecula with areas of newly formed tissues separates the deciduous tooth from the subjacent tooth germ. It is known that the resorption of the deciduous teeth and surrounding bone tissue during the development and eruption of the permanent teeth is an intermittent process with alternating resorption and apposition of hard tissues (21). The observation of newly formed areas of bone tissues underneath the deciduous tooth might thus possibly indicate a break in the resorption process.

SUMMARY

This investigation was carried out in order to get further information on the mineralized dental tissues. Two young normal dogs were injected, one with $Na_2S^{35}O_4$, the other with $Ca^{45}Cl_2$. From selected parts of the lower jaws thin ground sections were prepared. These sections were studied by means of a combination of autoradiographic and microradiographic techniques.

The autoradiographs revealed an uptake of both Ca^{45} and S^{35} in essentially the same locations of the bone and tooth tissues. There was thus a high uptake of isotopes in the periosteal areas and in young osteons and certain areas of the cancellous bone. In the deciduous teeth the labelling was most pronounced along the pulpo-dentinal border. In the tooth germs there was a high uptake of both isotopes along the pulpo-dentinal border in the same area. There was also a labelling along the enamel surface. In certain tooth germs, where the microradiographs indicated a high mineralization in the cusp tips, there was a diffuse labelling throughout the whole breadth of the enamel. These findings were interpreted as an expression of a secondary mineralization.

The uptake of S^{35} differed in certain respects from that of Ca^{45}. In the tooth germs there was thus an uptake of S^{35} along the dentino-enamel junction, and in the soft tissues, especially in the pulps of the tooth germs, there was also a marked labelling of S^{35} only. Further, the labelling at the pulpo-dentinal border showed features differing from the autoradiographs obtained with Ca^{45}. Thus the uptake of S^{35} at this site was concentrated in two closely adjacent bands. It was concluded that the uptake of Ca^{45} took place in the mineralized constituents of the tissues. However, as regards S^{35}, one part was probably incorporated in the crystal lattice of the minerals, another

being deposited with the organic matrix, presumably as mucopolysaccharides containing ester sulfuric acid. This latter conclusion refers to the uptake along the pulpo-dentinal border.

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MEMBRANE STRUCTURES OF CYTOPLASM AND MITOCHONDRIA IN EXOCRINE CELLS OF MOUSE PANCREAS AS REVEALED BY HIGH RESOLUTION ELECTRON MICROSCOPY

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High resolution electron microscopy on ultrathin sections makes it possible to study particles of macromolecular dimensions in cells. Therefore this technique has been applied in an attempt to analyze the morphology of synthetic activities in the pancreas cells, of the aggregation of proenzyme molecules into zymogen granules, and of the extrusion of zymogen granule material from the cells. The basis for an attack on such problems is a careful descriptive analysis of the structural organization of the pancreas cells.

Similar problems have been attacked using light microscopic techniques (1, 4, 5, 11). The limitations of the techniques used in these studies are, however, obvious.

This paper deals with a first part of a descriptive analysis of the structure of the exocrine cells of the pancreas of mice kept at normal laboratory conditions, starved for 24 hours or treated with injections of pilocarpine hydrochloride. In a second paper the structure and mutual relations of zymogen granules and Golgi apparatus will be examined from a descriptive point of view.

No clear-cut variations of the ultrastructural organization could be observed as due to different experimental conditions. This organization appeared rather stable with the exception of distortions due to fixation. The description presented, therefore, refers to the organization of the normal exocrine cells.

When examining in a preliminary way (12) the fine structure of the pancreas exocrine cells a highly organized structure of the cytoplasm was observed. A polarization optical analysis of living pancreas cells made it justifiable to assume that the structure observed in fixed cells in the electron microscope represented vitally preformed formations. Therefore, an extended study seemed justified.

The exocrine cells of the pancreas have earlier been studied by means of the electron microscope by several investigators. Dalton (2) seems to be the first to describe a lamellar structure of the pancreas cytoplasm. Palade (8) described in the ground substance of the exocrine cell cytoplasm densely arranged fibrillar or canalicular structures. Oberling, Bernhard, Gautier and Haguenau (7) described these structures as mainly fibrillar. A similar structure was also observed in the cells of the parotis gland (3). Sjöstrand (12) analyzing the structure of the cytoplasm at a better resolution than the authors mentioned above, described the cytoplasm of the exocrine pancreas cells as organized in membranes, arranged in pairs and oriented mainly parallel to the cell membrane. These membranes were also observed in material fixed by freeze-drying. A birefringence of the cytoplasm of the living cells was also found and the character of this birefringence corresponded to what could be expected from such a system of membranes.

MATERIAL AND METHODS

The pancreas from 15 adult brown mice weighing 15–25 g have been examined. The animals were killed by decapitation in a cold room at +2° C. It seems desirable, when possible, to avoid the use of anaesthesized animals, as anaesthesia and operation may produce changes regarding the function of the cells. Working very fast the abdominal cavity was opened, the duodenum with its mesentery containing a thin layer of pancreas cells was excised and spread out on a glass plate chilled to the temperature of the cold room. These manipulations took 10 to 30 seconds and aimed at as fast a cooling of the tissue as possible to reduce the effect of agonal enzymatic reactions on the cell structure. With cooled scissors about 0.5 mm broad ribbons of the very thin pancreas tissue were cut out and immersed into a cooled 1 per cent osmium tetroxide solution isoton and buffered at pH 7.2 with veronal-acetate, a modification of the osmium solution proposed by Palade (8). The osmium tetroxide solution with the tissue was gently stirred during the fixation by means of a laboratory shaker. The tissue was fixed for 4 hours at +2° C and then washed with balanced Tyrode's solution for one hour, dehydrated over 70 per cent, 95 per cent and absolute alcohol in the cold. The tissue was left in 70 per cent alcohol over night in most cases. The last changes of absolute alcohol were performed at room temperature. After changing to n-butyl methacrylate the tissue was embedded mainly according to Newman, Borysko and Swerdlow (6), the polymerisation being carried out at 45° C.

Ultrathin sections were prepared with the microtome constructed by Sjöstrand (13). Only the superficial layers of the pieces of tissue were cut because the quality of the fixation is poor when getting below an about 40 μ thick surface layer (10, 17). The sections were collected on 20 per cent ethyl alcohol as liquid in the trough and picked up on formvar film covered copper grids. The formvar films were made from 0.25 per cent formvar in ethylene dichloride.

The sections were examined in an RCA EMU 2 c electron microscope at a low beam intensity without dissolving or subliming the methacrylate. The objective lens of the microscope was kept carefully compensated and clean. An aperture with a 120 μ

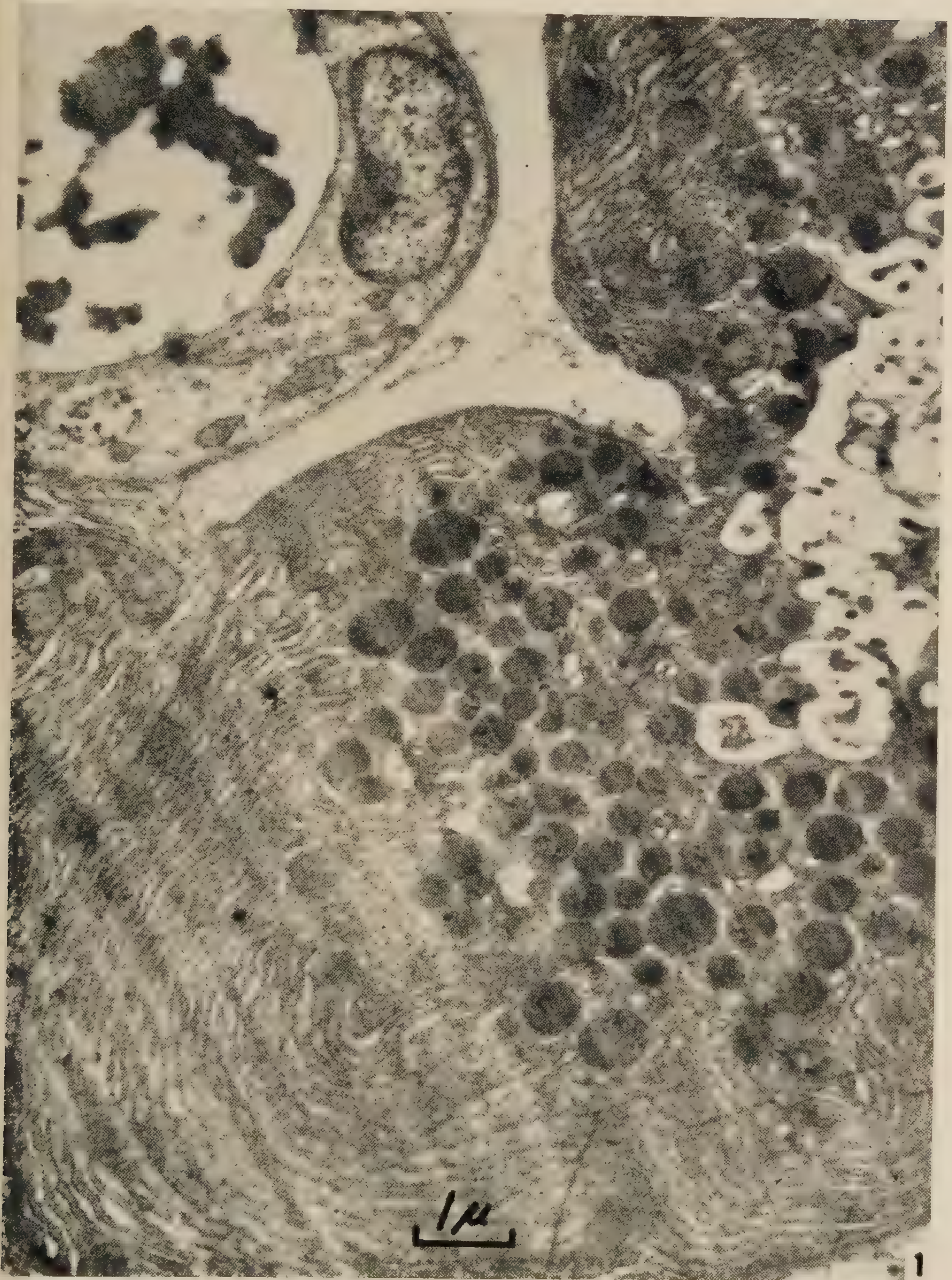

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Fig. 1. Survey picture of exocrine cells of the mouse pancreas. Along the upper part of the right side a small excretory duct. In the upper left corner a larger excretory duct partly separated from the exocrine cells through a lymph space. Magnification: 13,000 ×.

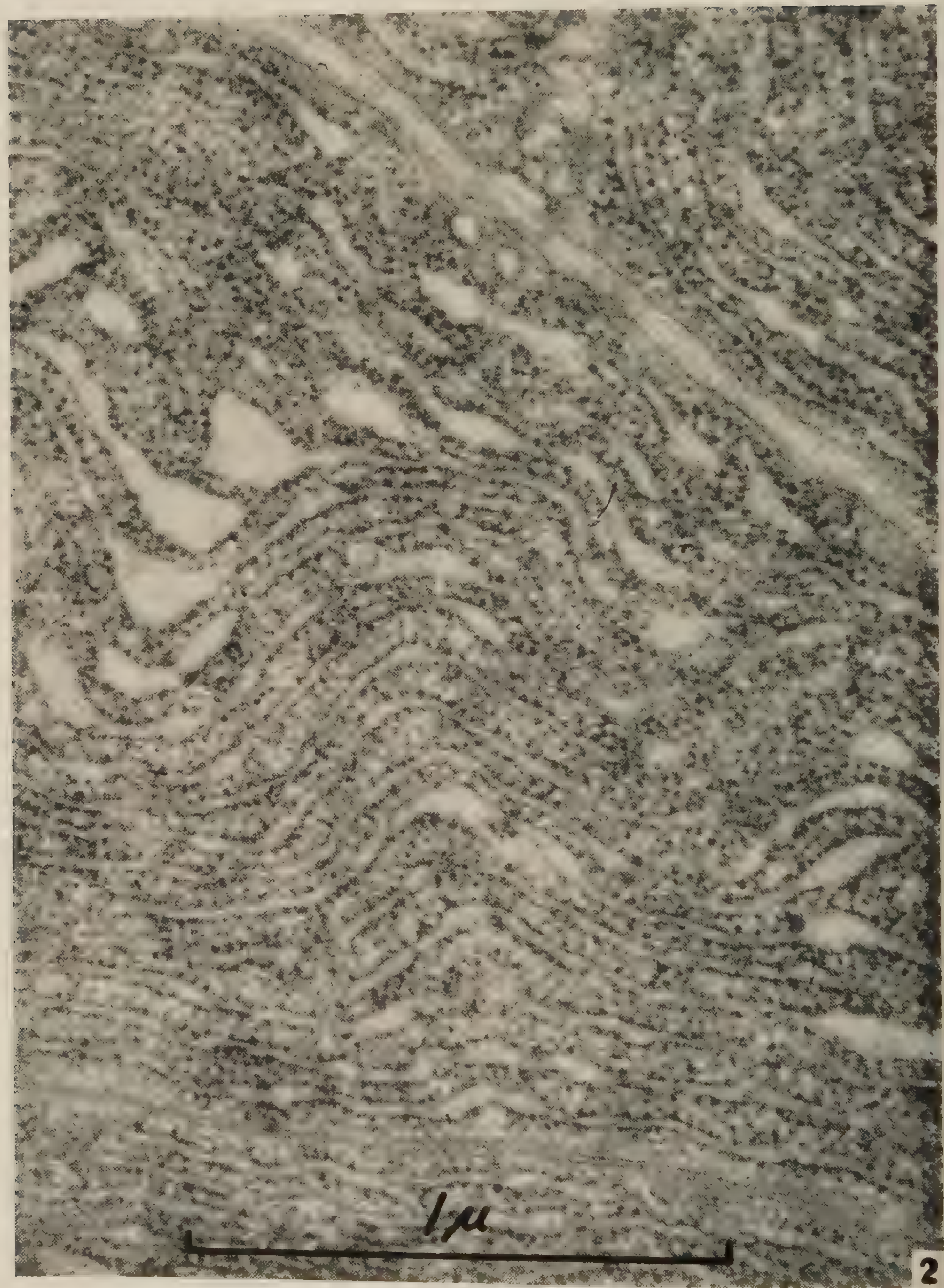

Fig. 2. Cytoplasm of three exocrine pancreas cells. In the upper part of the picture the cells
are separated by basement membranes in addition to the cell membrane. In the lower part cell
membranes only separate the cell territories. Magnification: 73,000 ×.

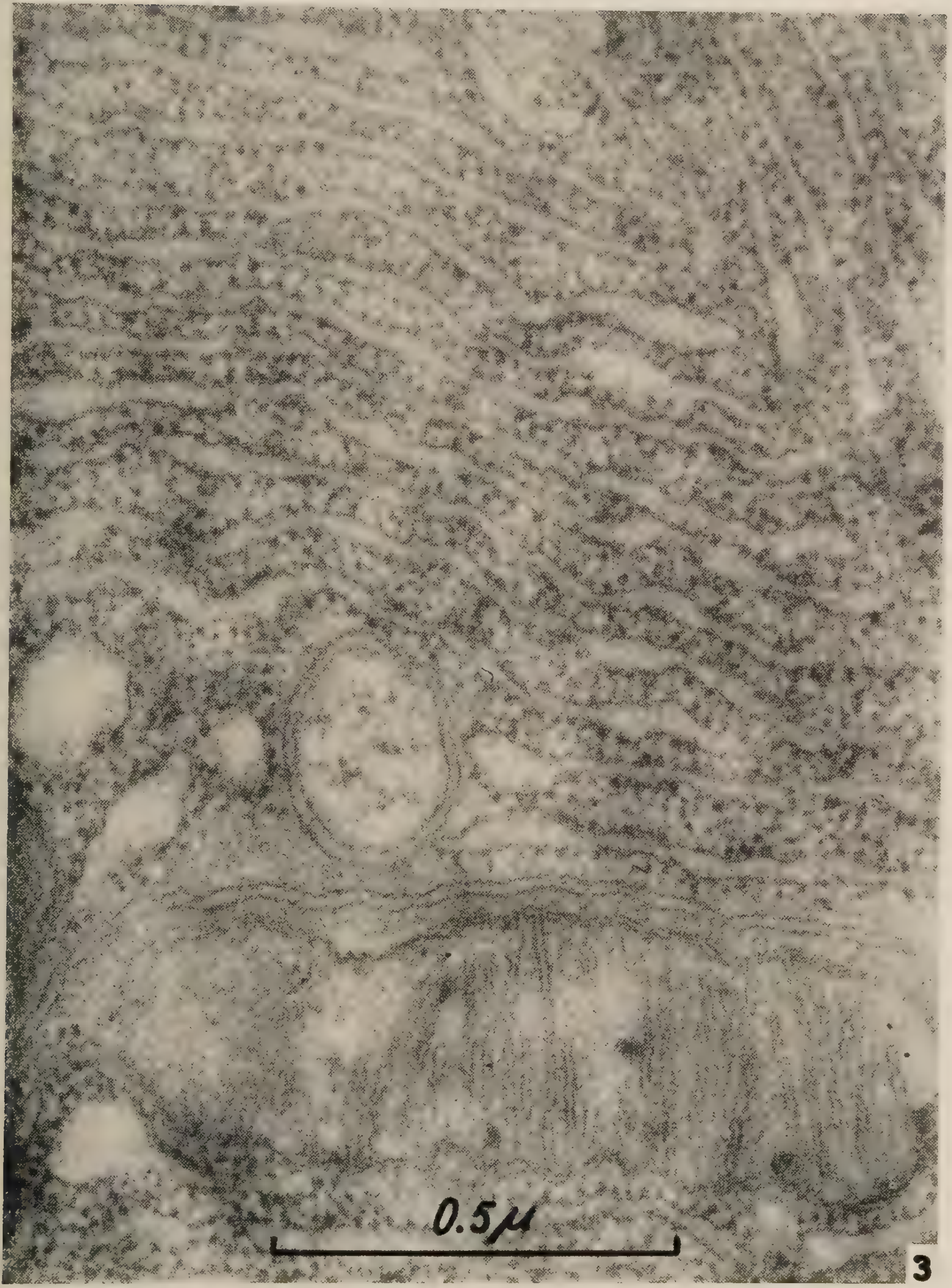

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Fig. 3. Intracellular cytoplasmic membranes arranged in pairs. In the lower part of the picture the cell membranes of two adjacent cells are observed and close to the cell border a mitochondrion is seen. Magnification: 108,000 ×.

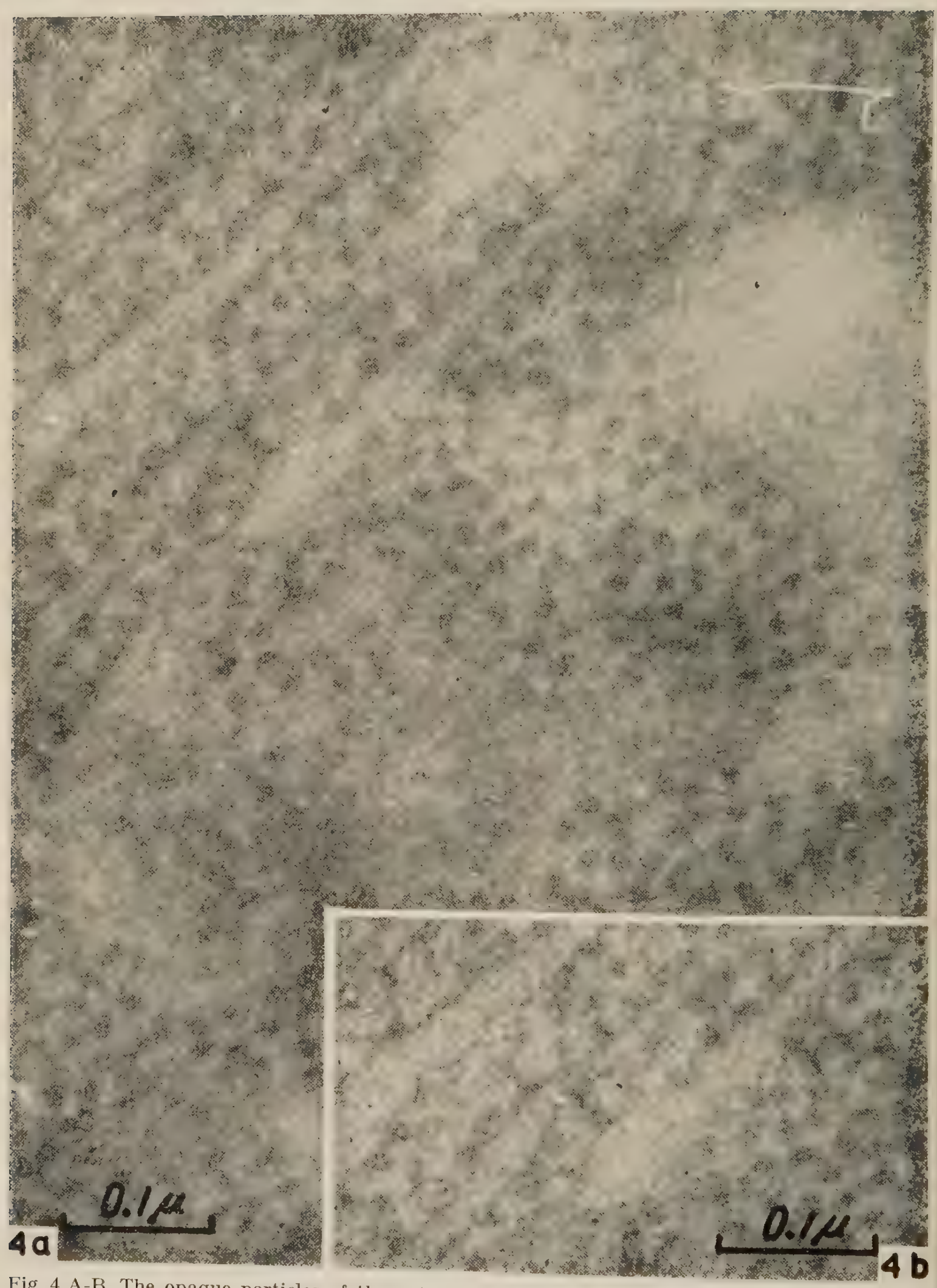

Fig. 4 A-B. The opaque particles of the cytoplasmic membranes at high magnification. In the upper left corner of Fig. 4 A the membranes are cut perpendicularly, in the rest of the field they are cut tangentially. The angular, irregular form of the particles is seen. Fig. 4 A is a close-to-focus picture. The underfocussed Fig. 4 B selected to demonstrate the dimensions of the background granularity as a function of the degree of underfocussing. Magnification: 210.000 ×.

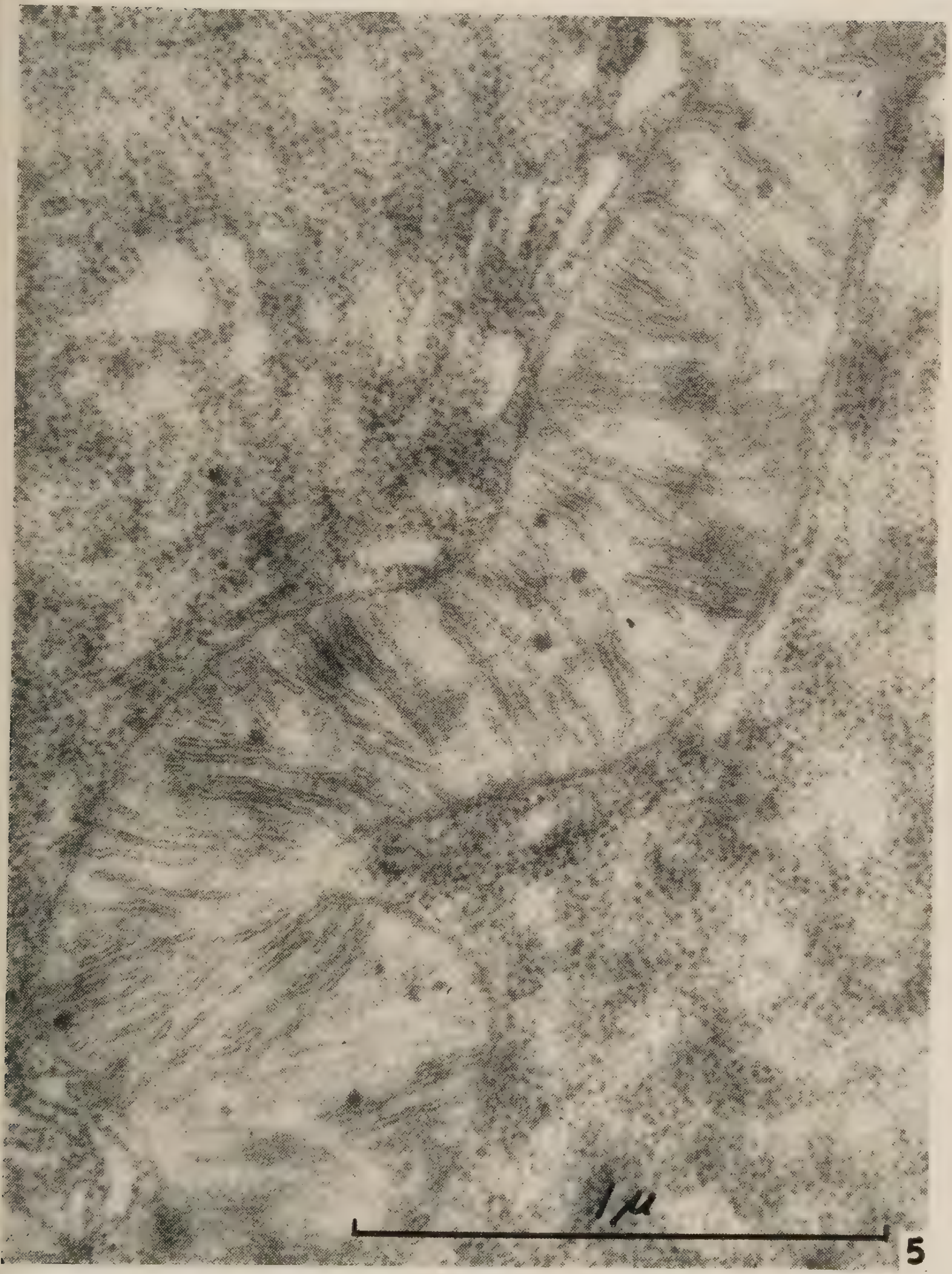

Fig. 5. Mitochondrion in exocrine pancreas cell bounded by a double lined surface membrane and with internal mitochondria membranes. The cytoplasmic membranes surrounding the mitochondrion are cut obliquely. Magnification: 73,000 ×.

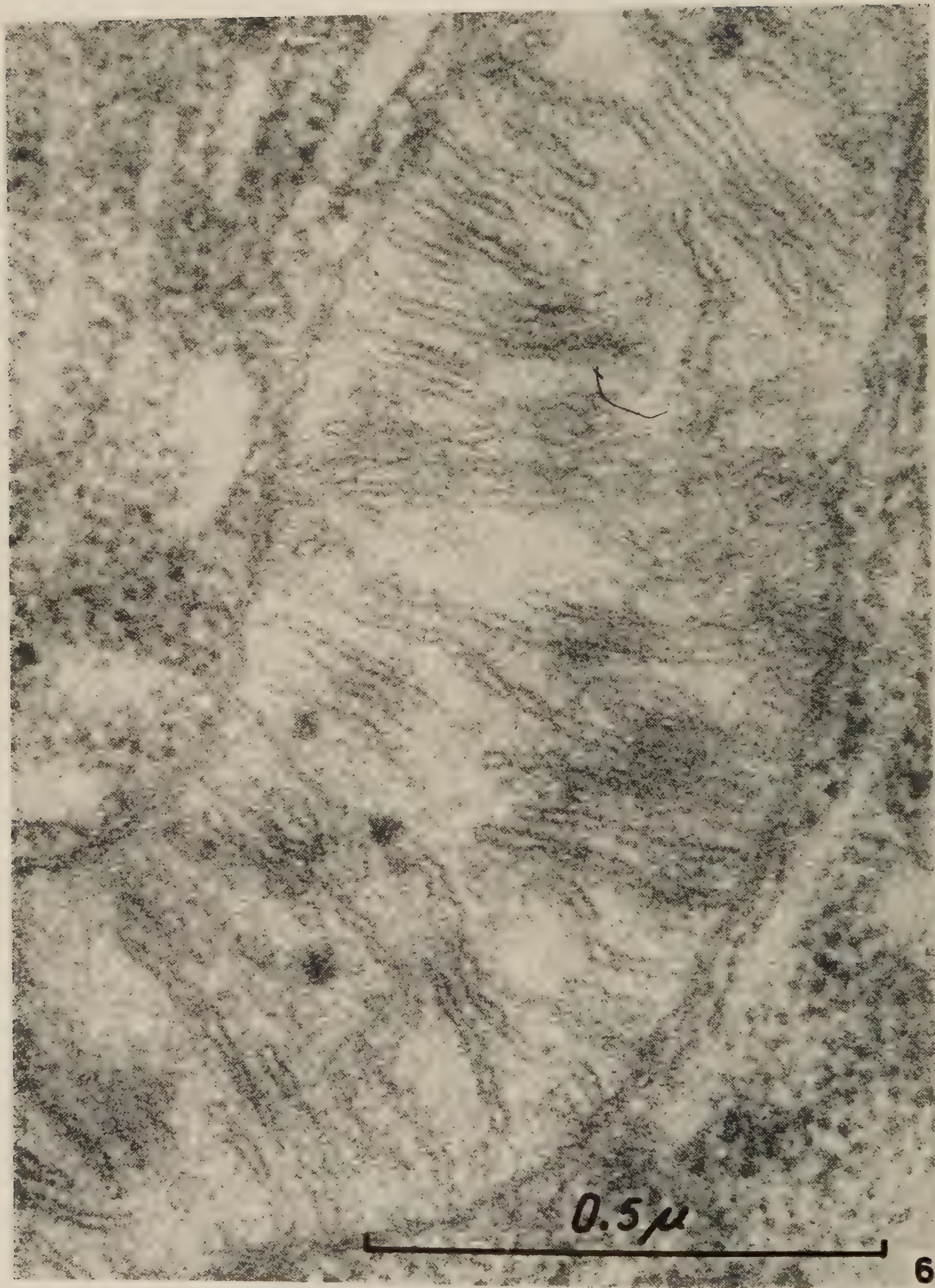

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Fig. 6. Higher magnification of part of Fig. 5. Magnification: 140,000 ×.

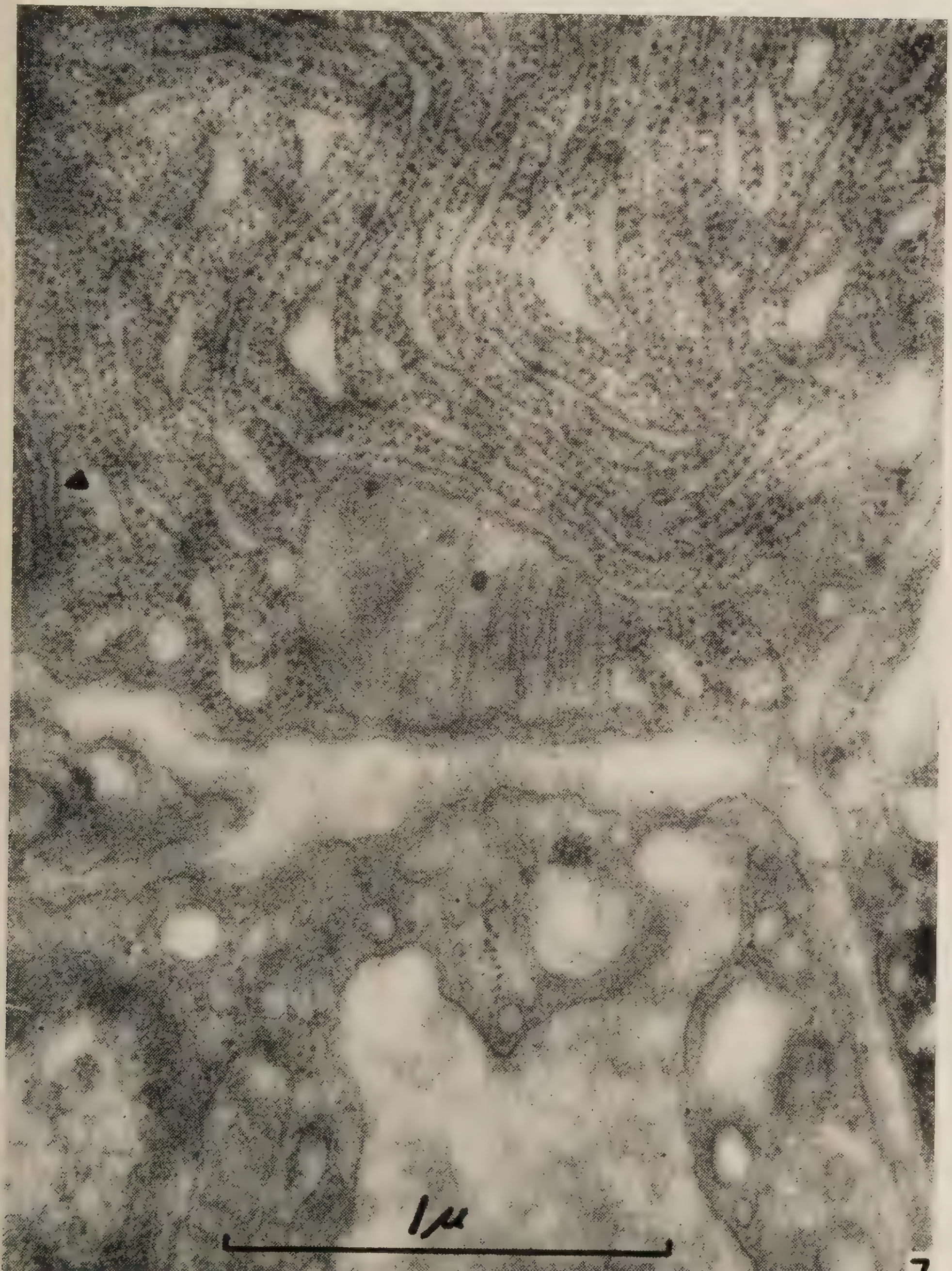

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Fig. 7. Exocrine pancreas cell facing a blood capillary. The exocrine cell as well as the endothelial cell are bounded by a cell membrane and a basement membrane that does not appear sharply outlined. Magnification: 60,000 ×.

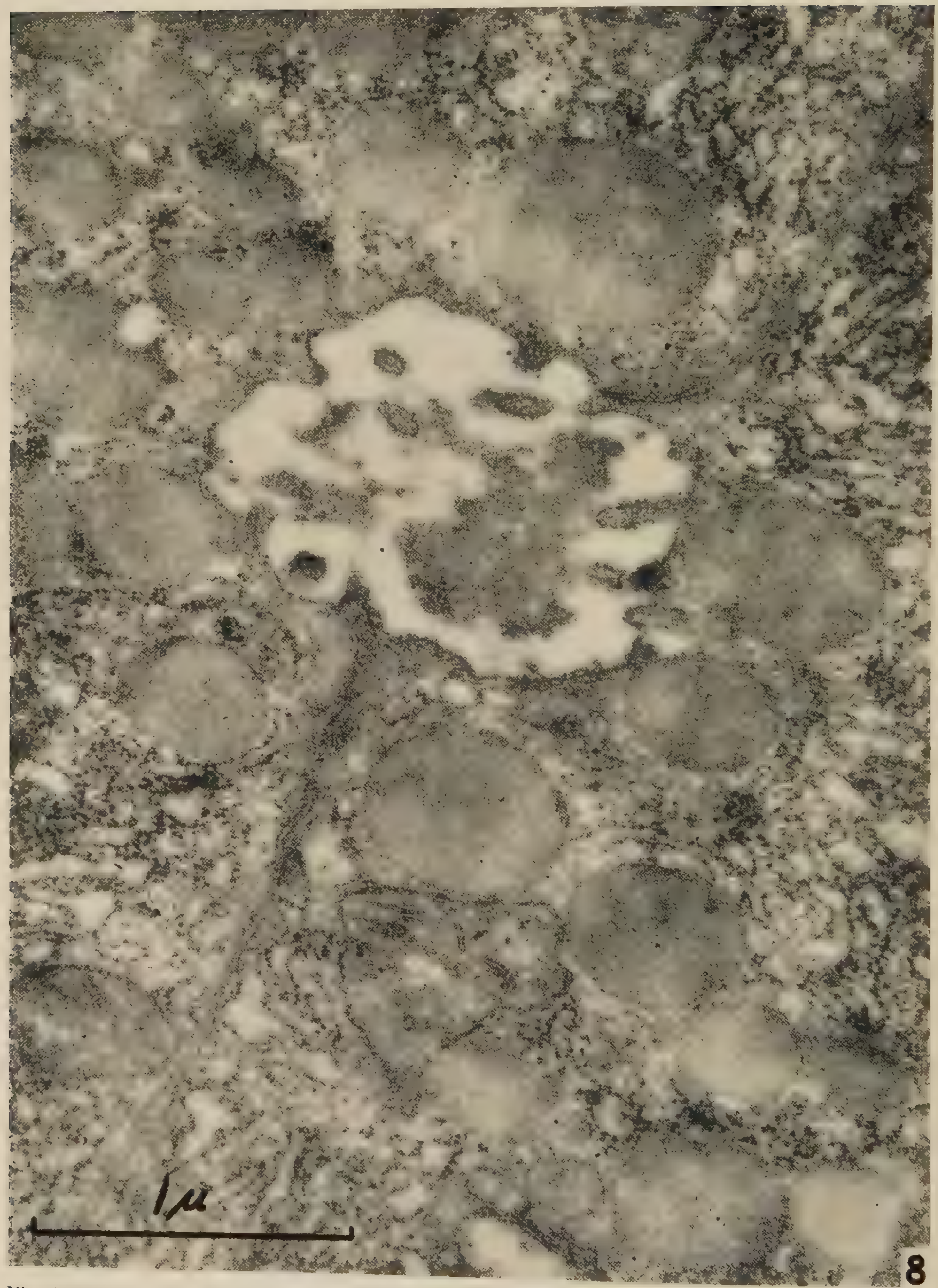

Fig. 8. Excretory duct lined by two cells. The cell borders are seen and close to the duct lumen the cytoplasm at the cell membranes appears homogeneous and rather opaque. Zymogen granules are seen surrounding the duct and in the lower part one mitochondrion is present. Magnification: 44,000 ×.

wide hole was used in the objective lens and three apertures according to Hillier were inserted in the projector lens pole piece. The centerable condensor aperture had a reduced diameter of the bore.

The magnification was calibrated as described by Sjöstrand (14) by means of a replica of a 1152 lines per mm grating and in addition latex particles, 58 G lot 3484. Both these test objects gave identical values for the magnification.

RESULTS

There are no difficulties in becoming oriented when examining the electron microscopic picture of the exocrine pancreas cells. A survey picture (Fig. 1) reveals the main structures with which we are acquainted from light microscopy. Already at this low magnification it is possible to observe the ground substance of the cytoplasm as organized in concentrically arranged, tightly packed membranes. In regions where these membranes are cut perpendicularly they appear as narrow stripes. When cut obliquely the stripes broaden. The membranes are separated at the sites of the mitochondria and the zymogen granules. The cell nuclei show a granular appearance. The cells are bordered by a thin cell membrane and towards the connective tissue, the lymph spaces, and the blood capillaries the cell membrane is associated with a basement membrane. Adjacent exocrine cells are mainly separated only by their cell membranes and a minute space in between the osmium stained parts of these membranes. The basement membrane sometimes is interposed between the exocrine cells, now and then accompanied by extracellular collagen fibril bundles. The most minute excretory ducts are bounded by the cell membranes.

The intracellular cytoplasmic membranes consist of a thin basic membrane with small opaque particles attached to the one side (Figs. 2–4). These particles show a varying form, from irregularly rounded to rectangular or triangular, the one side facing the basic membrane. The mean diameter of these particles is 140 Å (Table I). They are fairly regularly distributed over the surface of the basic membranes with a distance of 150–450 Å between their centers. The mean thickness of the basic membranes is 40 Å (Table II).

As most of the particles seem to be attached to the basic membrane we consider them as constituents of the intracellular cytoplasmic membranes which, therefore have one smooth and one rough or knob-covered side.

The smooth surfaces of the intracellular cytoplasmic membranes face each other giving the impression of the membranes being arranged in pairs. They always face the mitochondria, the zymogen granules and the cell membrane with their rough side but the cytoplasmic membranes adjacent

TABLE I

The dimensions of the opaque particles of the cytoplasmic membranes. Maximum and minimum diameters measured at mutually perpendicular directions. The measurements have been performed on one cell in each animal. 25 particles measured in each cell.

Animal No.	Max. diameter in Å		Min. diameter in Å	
	Mean	σ	Mean	σ
1	136±5	23	111±4	19
2	140±5	24	120±4	20
3	145±6	30	120±7	35
4	162±4	20	128±3	13
5	194±5	24	161±4	21
Mean	150		130	
Average diam.	140			

TABLE II

The thickness of the basic membrane of the cytoplasmic membranes. The measurements refer to one cell in each animal. 25 measurements in each cell.

Animal No.	Mean thickness in Å	σ
1	39±2	10
2	53±3	14
3	46±3	15
4	48±2	11
5	36±2	9
Mean	40	

to the nuclear membrane always have the smooth surface directed towards the nuclear membrane. Therefore at least one of the membranes appears as a single membrane.

In the sections the cytoplasmic membrane pairs may be followed for rather long distances without interruptions. At points scattered all over the cytoplasmic area at random or according to a certain pattern, the membrane pairs end abruptly. The two membranes of a pair then join closing the space in between the smooth sides of the membranes (Fig. 3). The space between the dotted sides of the membranes, on the other hand, are mutually connected by fairly narrow communications, oriented perpendicularly to the planes of the membrane pairs and in between their edges. Therefore, the spaces with the opaque particles appear continuous all through the cytoplasm of the cell.

The distance between the membranes varies considerably and in the sections the membranes show a wavy course. The distance between the membranes is on the side with the opaque particles generally 150–1,000 Å and between the smooth sides 70–700 Å. This latter space in between the smooth surfaces is frequently found rather wide with a material of low electron scattering power filling the gap between the membranes (Fig. 2). The width of this space varies with the quality of the fixation. When less well fixed the membranes are split apart and empty spaces of varying width are formed.

The membranes appear tightly packed and the number of pairs of cytoplasmic membranes between the cell membrane and the nucleus varies between 5 and 40 depending on the amount of cytoplasm separating the nucleus from the cell membrane.

When the cytoplasmic membranes are cut obliquely or are oriented parallel to the plane of the section the basic membrane does not show up distinctly or is not visible at all due to its low electron scattering capacity. Then, only the opaque particles are observed and the cytoplasm appears as consisting only of this component (Fig. 4).

The mitochondria. The rod shaped mitochondria frequently showing a wavy form are scattered all over the cells apparently at random. They are bordered by an outer double edged membrane (Figs. 3, 5–7). The two dark lines appearing in the EMG:s and presumably representing two components of the membrane are separated by a fairly constant distance. The mean distance when measured between the centers of the two dark lines is 110 Å. As the mean total thickness of the surface membrane is 140 Å the calculated thickness of the individual dark lines will be 35 Å and the width of the less opaque space in between the two lines will be 70 Å (Table III).

TABLE III

The dimensions of the outer mitochondria membranes. 25 measurements performed on each mitochondrion. Each animal is represented by one mitochondrion.

Animal No.	The total thickness in Å		The distance between the centers of the two dark lines in Å		Calculated thickness of the single lines in Å	Calculated width of the space between the two lines in Å
	Mean	σ	Mean	σ		
1	164±7	34	124±6	30	40	84
2	138±5	24	103±5	23	35	63
3	136±5	23	102±4	22	34	68
4	128±5	25	100±4	20	28	72
5	150±5	23	112±4	20	38	74
Mean	140		110		35	70

In the interior of the mitochondria densely packed inner membranes or plates are seen mainly oriented perpendicularly to the long axis of the mitochondrion. These inner membranes also appear double edged. One end of the membranes is in contact with the outer surface membrane and the other end in most cases is free from this membrane. The two dark lines meet at both ends of the membrane thus delimiting a narrow less opaque space in between the two lines. Sometimes the two lines of the inner membranes make individual contacts with the uninterrupted inner line of the outer surface membrane. There is with few exceptions no continuity observed between the central space in the inner and the outer membranes. In the sections the length of the internal mitochondria membranes were estimated relative to the diameter of the mitochondrion. Of 111 inner membranes in 5 mitochondria examined, 5 per cent did not reach more than $^1/_4$ of the mitochondria diameter, 28 per cent reached $^1/_4$–$^1/_2$, 27 per cent $^1/_2$–$^3/_4$ and 40 per cent more than $^3/_4$ of the mitochondria diameter.

The mean distance between the centers of the two dark lines of the inner mitochondria membranes was 130 Å, their total thickness was 170 Å. The calculated mean thickness of the individual dark lines was 40 and the mean width of the less opaque space in between the lines was 80–90 Å (Table IV). The distance between the inner mitochondria membranes generally varies from 70 to 400 Å.

In between the inner membranes opaque spherical bodies measuring 180–400 Å in diameter are observed.

The cell nuclei. The nuclear material consists of particles of uniform size with a mean diameter of 170 Å (Table V). These particles appear irregularly arranged and are more densely packed at the nuclear membrane and in one or a few denser areas in the interior of the nucleus. At high resolution these particles show an internal structure indicating that they consist of aggregates of still minor particles.

The nuclear membrane appears as a single thin line.

The cell membrane is observed as a single line bounding the cytoplasm (Figs. 1–3, 7–8). There are no indications of a zone of exoplasm adjacent to the cell membrane. The mean thickness of this cell membrane line is 60 Å (Table VI). When two exocrine cells are in close contact (Figs. 2, 3) the distance between the cell membrane lines of the two cells is fairly constant. The mean width of the less opaque space between these lines was measured to 110 Å. Cytoplasmic processes from one cell frequently protrude into an adjacent cell which explains some structural relationship observed in Fig. 3.

The basement membrane. The proximal or basal surface of the exocrine

TABLE IV

The dimensions of the inner mitochondria membranes. 25 measurements performed on each mitochondrion. Each animal is presented by one mitochondrion.

Animal No.	The total thickness in Å		The distance between the centers of the two dark lines in Å		Calculated thickness of the single lines in Å	Calculated width of the space between the two lines in Å
	Mean	σ	Mean	σ		
1	206±5	26	152±5	24	54	98
2	168±6	32	125±5	25	43	82
3	170±5	23	126±4	20	44	82
4	144±4	20	109±4	20	35	74
5	153±4	20	116±4	22	37	79
Mean	170		130		40	80

TABLE V

The dimensions of the opaque particles of the nucleus, measured as maximum and minimum diameters oriented mutually perpendicularly. 25 particles measured in each nucleus.

Nucleus No.	Max. diameter in Å	σ	Min. diameter in Å	σ
1	146±5	25	114±5	25
2	208±8	39	174±8	39
3	184±7	34	153±6	31
4	190±8	40	154±7	33
5	207±8	41	172±7	34
Mean	190		150	
Average diameter		170		

TABLE VI

The thickness of the cell membrane and the width of the space between the opaque component of adjacent cell membranes.

Cell No.	Number of measurements	The thickness in Å		The width of the space in Å	
		Mean	σ	Mean	σ
1	25	53±3	15	92± 6	31
2	25	63±2	11	114±12	60
3	25	43±2	10	146± 6	29
4	14	68±2	9	96± 7	26
5	11	76±6	20	115± 7	22
Mean		60		110	

cells is covered by a basement membrane which without interruption continues from cell surface to cell surface (Figs. 1 and 7). The surface of the basement membrane facing the interstitial tissue for instance a capillary wall (Fig. 7) appears rough and in the sections this surface is not presented by a sharply marked contour.

The thickness of the basement membrane generally varies between 150 to 400 Å presumably mainly due to variations as regards the cutting angle. A thin space may sometimes be seen between the cell membrane line and the basement membrane.

The excretory ducts. The wall of the smallest ducts consists of the cell membrane of the bordering exocrine cells without any visible reinforcement (Fig. 8). Close to the free surface of the cell the cytoplasm adjacent to the cell membrane shows a pronounced opacity (Fig. 8). The cell membrane shows fingerlike processes protruding into the duct lumen. The dimensions of these processes are about 800 × 3,000 Å. The amount of such processes varies considerably, sometimes they are lacking completely.

The surface of these processes frequently appears finely granulated presumably due to material adhering to the cell membrane.

Sometimes a zymogen granule may be found located directly under a protuberance of the cell membrane, separated from the duct lumen only by this membrane. In the duct lumen minute, irregularly formed particles representing the secreted material are observed but no zymogen granules.

The stability of the structures observed. The stability of the ultrastructural organization has been studied in connection with some experimentally induced changes of the cell activity. Furthermore the artefacts due to post mortal changes have been analyzed. The cell activity has been experimentally altered by the injection of pilocarpine hydrochloride and by starvation. On 14 mice pilocarpine hydrochloride was injected in doses of 0.04 and 0.5–1mg. The smaller dose was injected four times with 30 min. intervals according to Hirsch (4). In no case could any striking change of the ultrastructure of the exocrine cells be detected as compared to the tissue from animals killed under normal conditions.

Nine mice were killed after 24 hours starvation. Neither in these animals could any divergence as to the structural organization be observed.

To obtain information regarding how improper fixation might affect the ultrastructure of the exocrine pancreas cells the structure of these cells was analyzed in pieces of tissue fixed different times after the killing of the animal (17). Pieces of tissue that had been fixed 10 min. *post mortem* showed marked structural changes (compare Rhodin (10)).

Fig. 9 A-B. Schematic drawings presenting the basic observations made during this study.

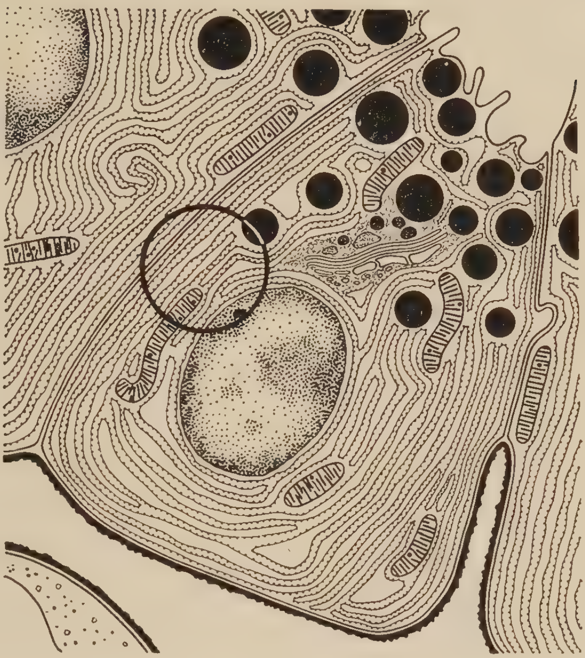

Fig. 9 A shows a whole exocrine pancreas cell with its basic constituents. The cytoplasmic membranes are not drawn with the right dimensions and number to make the drawing more clear.

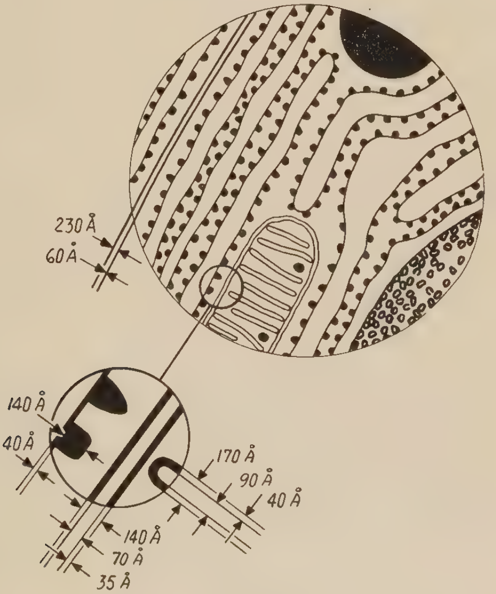

Fig. 9 B shows a magnification of the encircled area in Fig. 9 A and in the small circle the dimensions of cytoplasmic membranes and mitochondria membranes are presented.

The characteristic changes included a desorganization of the structure of the mitochondria combined with their swelling. The space between the smooth surfaces of the intracellular cytoplasmic membranes also widens to make the single membranes of a pair appear pushed aside and broken.

Similar pictures may be obtained from the inner parts of the tissue blocks even when the tissue was immersed in the osmium solution as quickly as possible after the killing of the animals and they then certainly reflect the slow penetration of the osmium tetroxide.

This distorted picture of the cytoplasmic membranes is similar to the observations described by Gautier and Diomede-Fresa (3) on the parotis gland cells after pilocarpine injections. From these observations they drew the conclusion that the cytoplasmic structure observed was related to the enzyme production of the cells and presumably in such a way that the membranes were directly transformed into the secretion product.

According to our experience the intracellular cytoplasmic membranes and the mitochondria structure represent constant and stable structures that until now have not been found to vary with the functional conditions of the cells.

A schematic drawing presented in Fig. 9 summarizes the observations made on the structure of the exocrine pancreas cells.

DISCUSSION

The intracellular cytoplasmic membranes. With few exceptions (2, 12) the characteristic cytoplasmic pattern of the pancreas exocrine cells has been interpreted as due to fibrillar or tubular structures (7, 9). Sjöstrand (12) interpreted this pattern as due to a system of intracellular cytoplasmic "double membranes". The present study has confirmed this latter interpretation. The arguments in favour of this assumption are for the first that there is statistically no probability for fibrils, canaliculi or tubules to be oriented parallel to the plane of the section over so long distances and in so many cells at the same time and never to appear cut across. Secondly the membranous character may be directly observed when the membranes are cut at an oblique angle. In the lower part of Fig. 1 the membranes may be followed for a long distance and in the lowest part of the picture they become more and more obliquely oriented with respect to the plane of the section. A third argument is the fact that when counting the number of membranes located in between the cell border and the nucleus, that number always is found to be uneven. The reason is that the membrane adjacent to the nuclear membrane lacks a partner. This fact is difficult to explain if assuming that we were dealing with tubes.

Already in 1880 (see Zimmermann, (19)) membranous structures were claimed by Heidenhain to exist in the exocrine pancreas cells. They were called "Basallamellen" or "ergostoplasma". These lamellae observed in the light microscope certainly represented aggregations of the intracellular cytoplasmic membranes as a result of the fixation. Although the term ergostoplasma connects the electron microscopic findings with observations made with the light microscope it should not be used because the term also includes an interpretation of its functional significance. It seems more justified to use a terminology that is purely descriptive and that presents the form and general morphologic character of the structure.

The dimensions, 140 Å, of the particles attached to the basic membranes of the cytoplasmic membranes represent macromolecular dimensions. At high resolution it is sometimes possible to observe a certain variation of the opacity in different parts of a single opaque particle. A granular structure or even star-shaped appearance of these particles may thus easily be observed in EMG:s that are underfocussed (Fig. 4 B). This appearance is due to a general background granularity presumably reflecting a structure of the methacrylate, the formvar film or the contaminating layer built up during the exposure of the specimen to the electron beam. The size of the granular elements of this background granularity is a function of the degree of underfocussing. In close to focus pictures (Fig. 4 A) the individual granules are extremely small and, in consequence, the opaque particles appear almost homogeneous. On the other hand the reduced contrast in close-to-focus pictures will reduce the chances to visualize differences regarding the opacity. For the time being no characteristic internal structure has been observed in the opaque particles in spite of an analysis at a resolution better than 20 Å. The reason might be that an internal structure, if present, is of an order of magnitude below the resolution of the EMG:s or that the contrast is too minute.

The characteristic angular form of the opaque particles is clearly visible also in close-to-focus pictures (Fig. 4 A).

The cytoplasmic membrane particles react very intensely with osmium tetroxide when judging from their opacity and from the fact that they did not appear in frozen-dried preserved material (12). These particles seem mainly to be attached to or in close contact with the basic membrane. It is, however, possible that opaque particles of exactly the same size and form may occur free between the membranes.

The cytoplasmic membranes are organized in pairs. The fixation and embedding may produce distortions of these membrane pairs. Especially the space between the smooth sides of the individual cytoplasmic membranes seem to be susceptible to *post mortem* effects and to fixation (17). The forces anchoring the cytoplasmic membranes to each other seem to be especially weak on the smooth side of the membranes. It, therefore, does not seem justified to assume that a continuous lipid layer should exist in this space. No indications of an oriented lipid layer has so far been found during the polarization optical analysis of the living pancreas cells. The birefringence of the cytoplasm is negative with respect to a direction perpendicularly to the cell surface.

The spaces between the smooth sides of the cytoplasmic membranes seem to represent closed and very thin compartments. The space with the opaque particles, on the other hand, seem to represent a continuous cytoplasmic "phase", which contains the mitochondria, the Golgi apparatus, the zymogen granules and which reaches the cell membrane.

The cytoplasmic membranes with the opaque particles represent the dominating structure of the cytoplasm of the exocrine pancreas cells. They represent a fundamental principle of cytoplasmic organization of these cells. This fact makes it justified to assume that they are structures of importance for the enzyme synthesis in these cells. The cell membrane, the mitochondria, the Golgi apparatus and the zymogen granules, which structures may be assumed to be of importance for the secretion process, show intimate relations to the opaque particles.

The mitochondria. The structure of the exocrine cell mitochondria is basically similar to the organization of mitochondria in kidney tubular cells (12, 18) and in the guinea pig retina (15, 16).

When examining the structure in detail there are characteristic differences observed between the three kinds of mitochondria, the pancreas, the kidney and the retinal rod mitochondria. In the kidney mitochondria most of the inner mitochondria membranes reach across the whole width of the mitochondrion, in the pancreas mitochondria, only 40 per cent of the inner membranes extend more than $^3/_4$ of the diameter of the mitochondrion and in the retinal rod inner segments most of them are even shorter.

The figures for the thickness of the double edged membranes show some differences. The outer surface membrane of the pancreas mitochondria is definitely thinner, 140 Å, than the inner mitochondria membranes 170 Å. A difference regarding the thickness of outer and inner membranes was observed in the retinal rod mitochondria but was considered too slight to be significant. In the kidney mitochondria no such differences could be observed. The thickness of the inner membranes in the pancreas and the mouse kidney and retinal rod mitochondria are very similar (170, 160, 160 Å).

No support has been found for the opinion of Palade (9) regarding the internal structure of mitochondria. There are no indications that the inner membranes represent folds of a single edged surface membrane. The inner membranes are individual structures with only topographic relations to the outer membrane. Therefore, the term, *cristae mitochondrales,* is misleading.

For the interpretation of the internal structure of the outer and inner mitochondria membranes it may be assumed that the double edged appearance is due to these membranes being composed of three layers. It might tentatively be assumed in accordance with Sjöstrand (16), Sjöstrand and Rhodin (18) that these layers correspond to one lipid layer between two protein layers, the latter being represented by the dark lines.

The cell nucleus. The nuclear membrane appears as a single dark line and therefore differs from for instance kidney tubular cell nuclei. With the exception of the fairly uniformly sized particles the minute structure of the nuclei does not show any high degree of organization.

SUMMARY

The ultrastructure of the exocrine cells of the mouse pancreas has been studied on ultrathin sections at a resolution better than 20 Å. The tissue was fixed in buffered (pH 7.2) isoton osmium tetroxide solution and embedded in n-butyl methachrylate.

The cytoplasm consists of concentrically arranged, densely packed, 40 Å thick membranes. To one side of such a basic membrane opaque particles are attached. The size of these angularly shaped particles is 140 Å and the distances between the particles in the plane of the basic membrane is 150–450 Å. The membranes with the particles are considered one structural unit and are called *intracellular cytoplasmic membranes.* They are arranged in pairs with the smooth sides facing each other. The cytoplasmic membranes face the cell membrane, the zymogen granules and the mitochondria with the particle-covered side but face the nucleus with the smooth side.

The mitochondria are bordered by an outer 140 Å thick double-edged membrane. The double-edged appearance is due to the membrane consisting of two osmium stained layers separated by a less opaque space. The thickness of the individual osmium stained layers is 35 Å.

In the interior of the mitochondria there are double-edged membranes or

plates oriented mainly perpendicularly to the long axis of the mitochondria. The mean thickness of these membranes is 170 Å and the thickness of the osmium-stained layers 40 Å.

The cell membrane is represented by a 60 Å thick line. When two cells are in close contact these cell membrane lines are separated by a 110 Å wide space.

The surface facing the interstitial tissue is covered by a continuous basement membrane. The thickness of this basement membrane is about 150 Å.

The most proximal parts of the secretory ducts are bounded only by the cell membranes of the surrounding exocrine cells. The free cell surface is characterized by fingerlike processes protruding into the duct lumen.

No definite changes of the ultrastructural organization were observed as the result of injections of pilocarpine hydrochloride or of starvation. Improper fixation, on the other hand, introduces drastic structural changes of mitochondria and intracellular cytoplasmic membranes. Therefore, it is necessary to be very cautious when considering structural changes as due to a variation of physiologic activity of the cells.

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ULTRASTRUCTURE OF GOLGI APPARATUS OF EXOCRINE CELLS OF MOUSE PANCREAS

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The Golgi apparatus as revealed by light microscopy appears as a morphologically ill defined structure the form of which differs appreciably and the size of which varies considerably. It is made visible only by unspecific staining methods and its demonstration in living normal cells is uncertain. There is some justification in assuming that what has been described as the Golgi apparatus in fact represents a variety of more or less unrelated cell structures.

In this state of affairs it seems important to be able to define in a more precise way the structure of the Golgi apparatus and to reveal its elementary components.

The present study has shown that the Golgi apparatus represents a well defined structure with a few basic elementary components. Only the electron microscope used at a fair resolving power can reveal this organization. What the light microscope has contributed is merely a pointing to the spot in the cell where the Golgi apparatus or frequently some other cell structure were located.

The light microscopy of the Golgi apparatus has been reviewed by Kirkman and Severinghaus (8, 9), Hirsch (7), Hibbard (6) and Bourne (2). Dalton and Felix (4) discuss some of the most recent papers.

Only a few papers (3, 4) are dealing with electron microscopic studies of the Golgi apparatus. Dalton and Felix made a comparative study with phase microscopy and electron microscopy of the Golgi apparatus in the epithelial cells of the duodenum and of the head of the epididymis of the mouse. The electron microscope revealed the Golgi apparatus as consisting of "numerous cytoplasmic strands outlining small vacuoles many of which are too small (0.05 μ) to be revealed with the light microscope". The resolution of the EMG:s published was however not sufficient to demonstrate the elementary components of the Golgi apparatus. Recently the same authors (5) described the Golgi material as consisting of "vacuoles marginated by lamellae comparable to those of the basophilic substance".

MATERIAL AND TECHNIQUE

The observations reported in this paper have been made on the same material as that used for the previous report (12) on the ultrastructure of the cytoplasm and mitochondria of the exocrine cells of the mouse pancreas. All essential data regarding material and technique are presented in that paper and are therefore omitted here.

RESULTS

The location of the Golgi apparatus. In practically all exocrine cells that have been sectioned through the distal half of their cell body characteristic zones of cytoplasm have been observed which show a striking difference as to morphology when compared to the rest of the cytoplasm which has been described in our previous paper (12). These cytoplasmic zones appear only in the distal part of the exocrine cells and show intimate topographic relations to the zymogen granules. They are found at the proximal border of the zymogen granule accumulation and in between these granules (Fig. 1). They may be located close to the cell nucleus or along a cell border facing an adjacent exocrine cell but rarely extend to the free surface of the cell lining the excretory duct. These zones occupy a fairly large volume of the distal part of the cells. In sections they cover one or several elongated areas of irregular form.

The location of these zones corresponds to the site of the Golgi apparatus as described in light microscopy.

The basic structure of the Golgi apparatus. The Golgi apparatus as observed in the electron microscope appears as geometrically rather complicated. Among the structural elements that may be seen are thin membranes here called the *Golgi membranes,* arranged mainly in pairs, frequently lining irregularly formed vacuolar spaces. In addition to the membranes, granules of varying size, form, and opacity are observed. They will here be called the *Golgi granules.* The membranes and granules are embedded in a homogeneous ground substance here called the *Golgi ground substance* the opacity of which differs in different regions.

The Golgi membranes occur in groups each group being surrounded by Golgi ground substance and Golgi granules. Several Golgi zones are thus formed in the same cell. Judging from what can be seen on sections these Golgi zones are not necessarily continuous. Most observations point to the possibility that these zones may be completely separated by systems of cytoplasmic membranes partially mixed with zymogen granules.

The Golgi membranes (Fig. 2–3) occur in several groups each group consisting of mostly two to five tightly packed membrane pairs. The average thickness of these membrane pairs is 180 Å and the distance between the centers of the two constituent membranes is 130 Å. The thickness of a single membrane may be calculated to 60 Å and the width of the space in between the two membranes of a pair to 60 Å. These membranes are not in contact with any opaque particles and, therefore, they are easily distinguished from the cytoplasmic membranes, both their surfaces appearing smooth. The space separating the membrane pairs frequently shows a more pronounced opacity than does the space bounded by the two membranes of a membrane pair (Fig. 2). At the ends of the membranes as appearing in the sections the space separating the membrane pairs often is closed due to the fusion of two adjacent membranes, belonging to two neighbouring membrane pairs (Fig. 2).

Frequently fairly large spaces which appear like empty vacuoles, are observed in between the split membrane pairs (Fig. 1–3). In such places the membranes show a wavy and irregular course. Otherwise the membrane pairs are arranged roughly mutually parallel. The general orientation of the Golgi membranes is fairly parallel to the plane of the surrounding cytoplasmic membranes or perpendicularly to that plane.

The Golgi ground substance in which the Golgi membranes are embedded appears homogeneous, granulated or reticulated (Fig. 4). In this specialized cytoplasmic region no particles of the opacity and size characteristic of the cytoplasmic membrane particles are observed.

The Golgi granules representing dimensions from 40 Å in diameter to the size of zymogen granules frequently exhibit a more opaque surface zone and a less opaque central area (Fig. 5). This surface zone may show a more or less sharp boundary towards the Golgi ground substance. Sometimes this demarcation is rather diffuse. In other cases the opaque surface layer is very thin and appears as a thin surface membrane similar to that of the zymogen granules (Fig. 2).

A distinct surface layer may be restricted to a part of the circumference of the granules. In the region where an opaque surface layer is missing the material of the granules appears more or less continuous with the Golgi ground substance (Fig. 5–6).

The material of the granules may represent all degrees of opacity from that of the Golgi ground substance to that of the zymogen granules (Figs. 2, 3 and 5). The less opaque granules frequently show an internal quite irregular structure and may appear as an aggregation of small bodies partly with diffuse outlines (Fig. 6). Some granules show a reticulated or spotted pattern.

The zymogen granules are located to the distal part of the cells. They have a spherical form or appear slightly oval in the sections due to compression at the sectioning (Figs. 1–3). In thicker sections they stand out well due to a pronounced opacity. In ultrathin sections their opacity is less than that of the cytoplasmic membrane particles. They then show a more opaque, very thin surface layer, presumably representing a bounding membrane (Fig. 3). The structure of the granules is mainly homogenous. Local variations regarding the opacity of the granular material may be observed.

The relations between the intracellular cytoplasmic membranes and the Golgi ground substance. The cytoplasmic membranes always face the Golgi ground substance with their rough, dotted surface. It seems possible that some opaque cytoplasmic particles most adjacent to the Golgi ground substance have no contact with a basic membrane but are embedded in the outermost zone of the Golgi ground substance. A precise judgement is difficult as it is necessary to exclude that this picture is not due to the cytoplasmic membrane at the edge of the Golgi zone being cut obliquely. When the system of cytoplasmic membranes run perpendicularly to the long axis of the elongated Golgi zone they end abruptly with the space between their smooth sides closed by their basic membrane.

The relation between the Golgi membranes and the Golgi granules. At the Golgi membranes small elongated bodies are frequently observed oriented with their long axis parallel to the membranes. The Golgi membrane pairs may appear divided into a series of elongated bodies lined up in more or less parallel rows (Fig. 5). The interior of these bodies is less opaque than the well marked surface layer (Fig. 1). This surface structure resembles the Golgi membranes regarding opacity and thickness and sometimes one side of the body is in intimate relation to the Golgi membranes.

Similar bodies but with a more rounded form are seen within the Golgi ground substance at varying distances from the Golgi membranes and represent one variety of the Golgi granules (Fig. 6).

The surface layer of the granules facing the Golgi membranes frequently is less well defined or shows a very irregular form with extensions into the ground substance sometimes reaching the Golgi membranes and thus connecting the granules to these membranes (Fig. 3–4).

The relations between the Golgi granules and the zymogen granules is very intimate. The latter granules are found at the periphery of, or even within the Golgi zone, then mostly in its peripheral region. The zymogen granules with the most intimate topographical relations are mostly small sized to medium sized and show a more or less irregular form (Fig. 7) in contrast to the nicely rounded form of the majority of the zymogen granules.

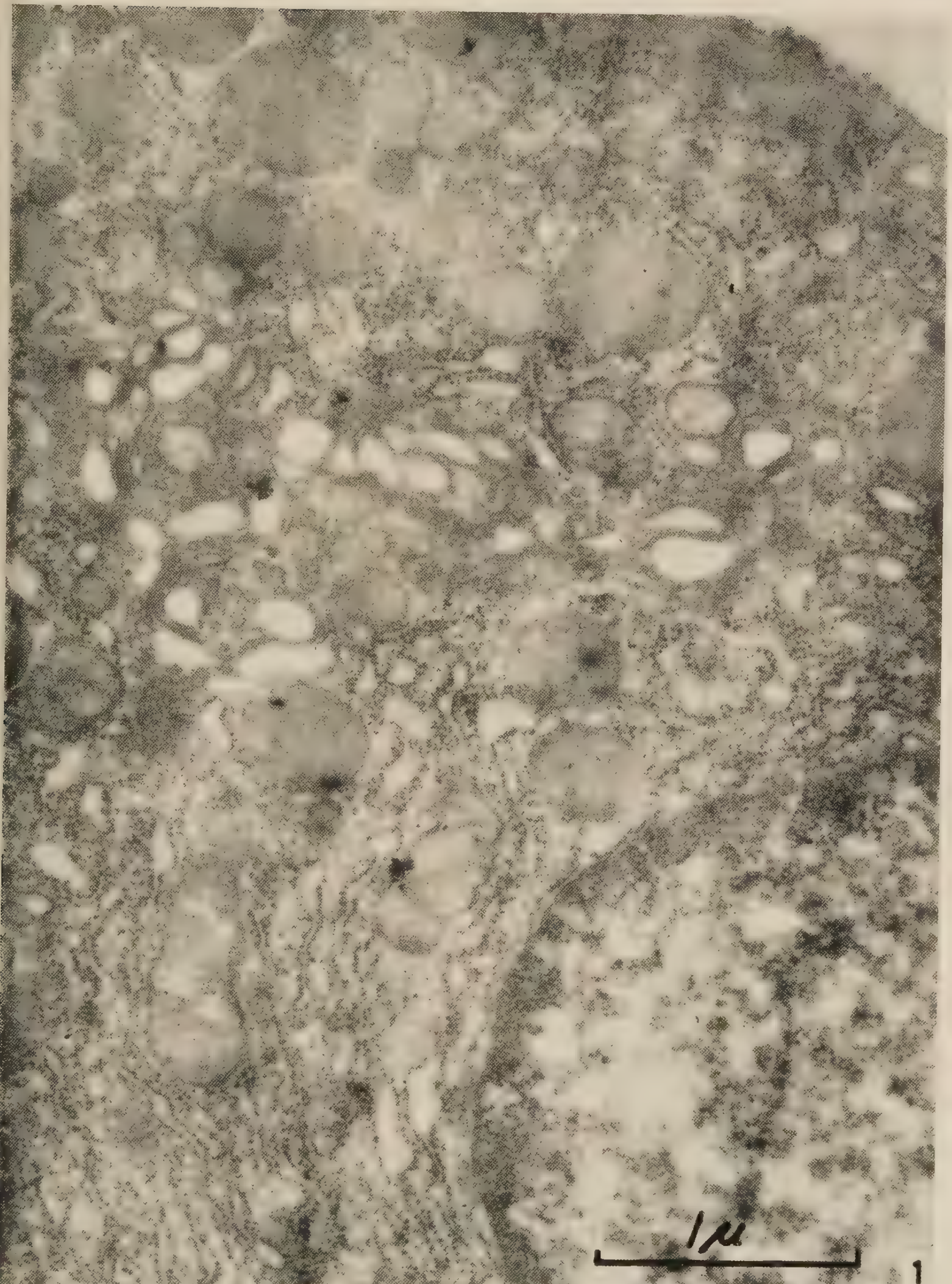

Fig. 1. Survey picture of exocrine pancreas cell showing the Golgi apparatus in the distal part of the cell body. In the upper right corner an excretory duct, in the lower right corner a cell nucleus, to the left of the nucleus two mitochondria embedded in intracellular cytoplasmic membranes. The Golgi zone is indicated by vacuolar spaces bounded by Golgi membranes. In the Golgi ground substance a great amount of Golgi granules. Around the Golgi zone scattered zymogen granules. Magnification: 35,000 ×.

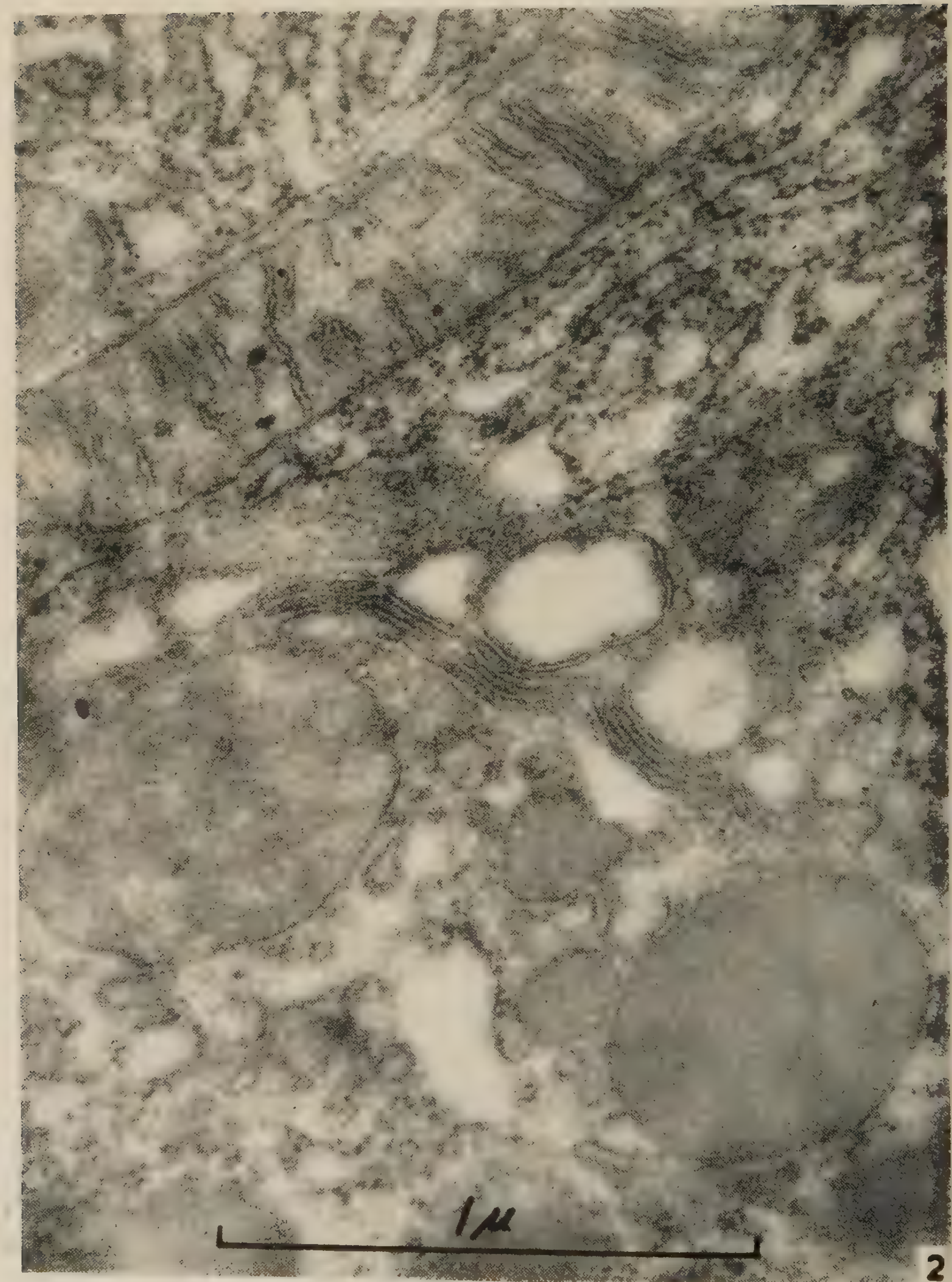

Fig. 2. Golgi apparatus with an aggregation of Golgi membrane pairs, partly bounding vacuolar spaces. In the right lower corner a zymogen granule. To the left side a Golgi granule with a marked dense surface layer, lower opacity and coarser structure than the zymogen granule. The surface of this granule shows irregular extensions towards the Golgi membranes. In the upper part of the picture a mitochondrion. Magnification: 73,000 ×.

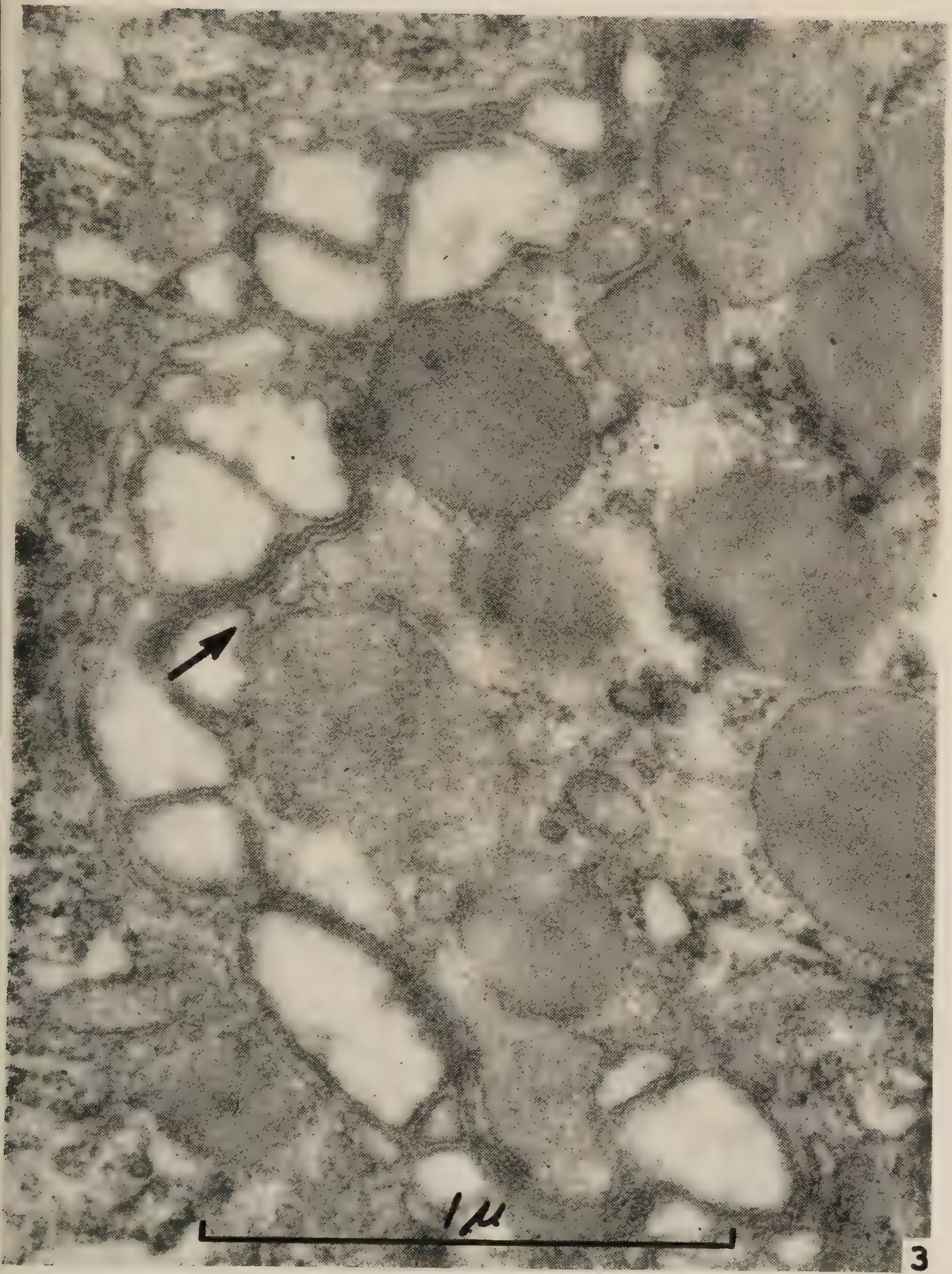

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Fig. 3. Golgi apparatus of exocrine cell of mouse pancreas. The Golgi membranes are bounding a series of vacuolar spaces. The granules to the right show a great variation as to size, form and opacity. The most opaque granules are characteristic zymogen granules. At the arrow a vaguely defined granule of low opacity and intimate relations to the Golgi membranes. Magnification: 73,000 ×.

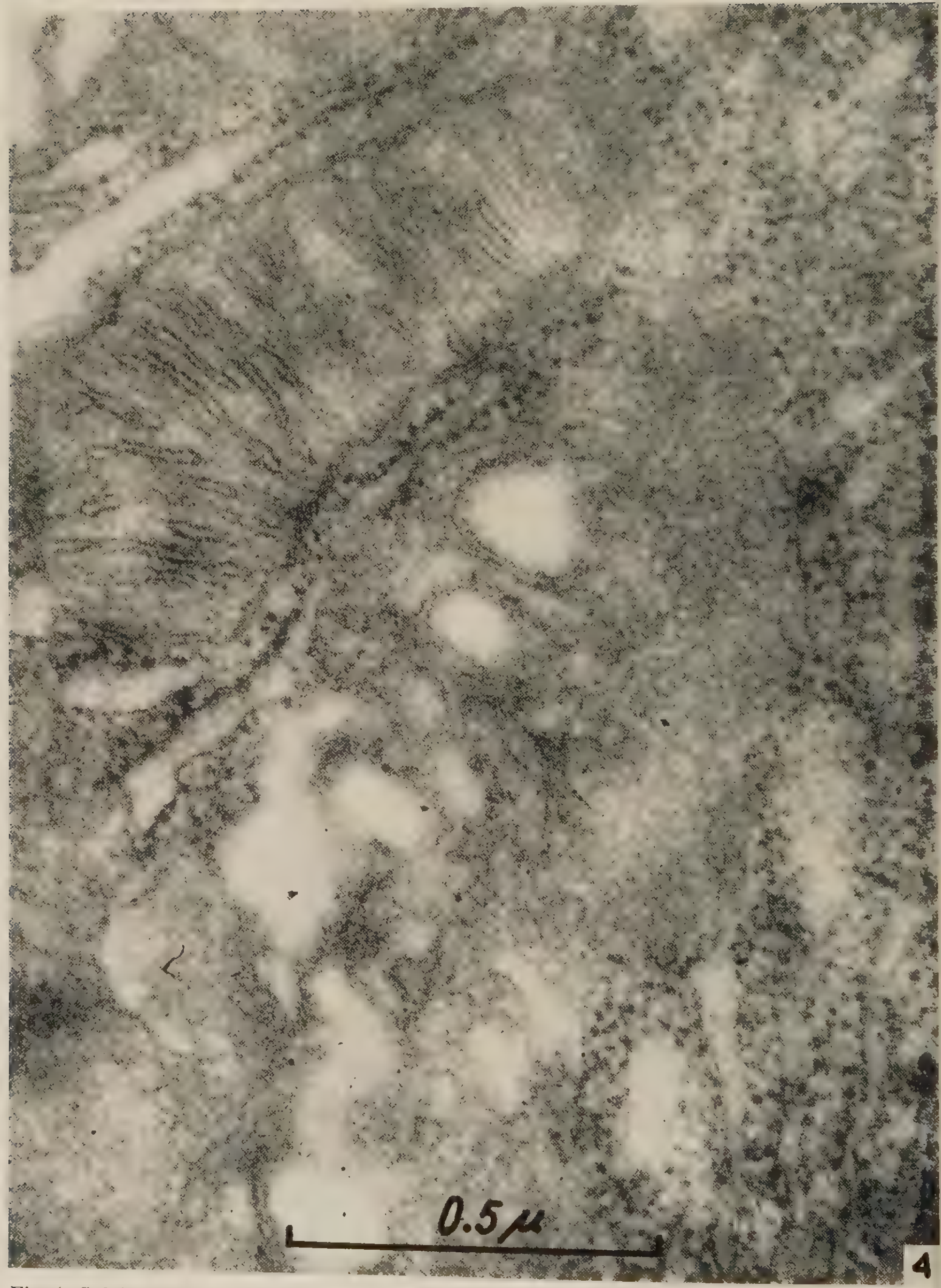

Fig. 4. Golgi ground substance with the border between the cytoplasmic membrane zone and a Golgi zone. In the upper left corner a mitochondrion. Magnification: 101,000 ×.

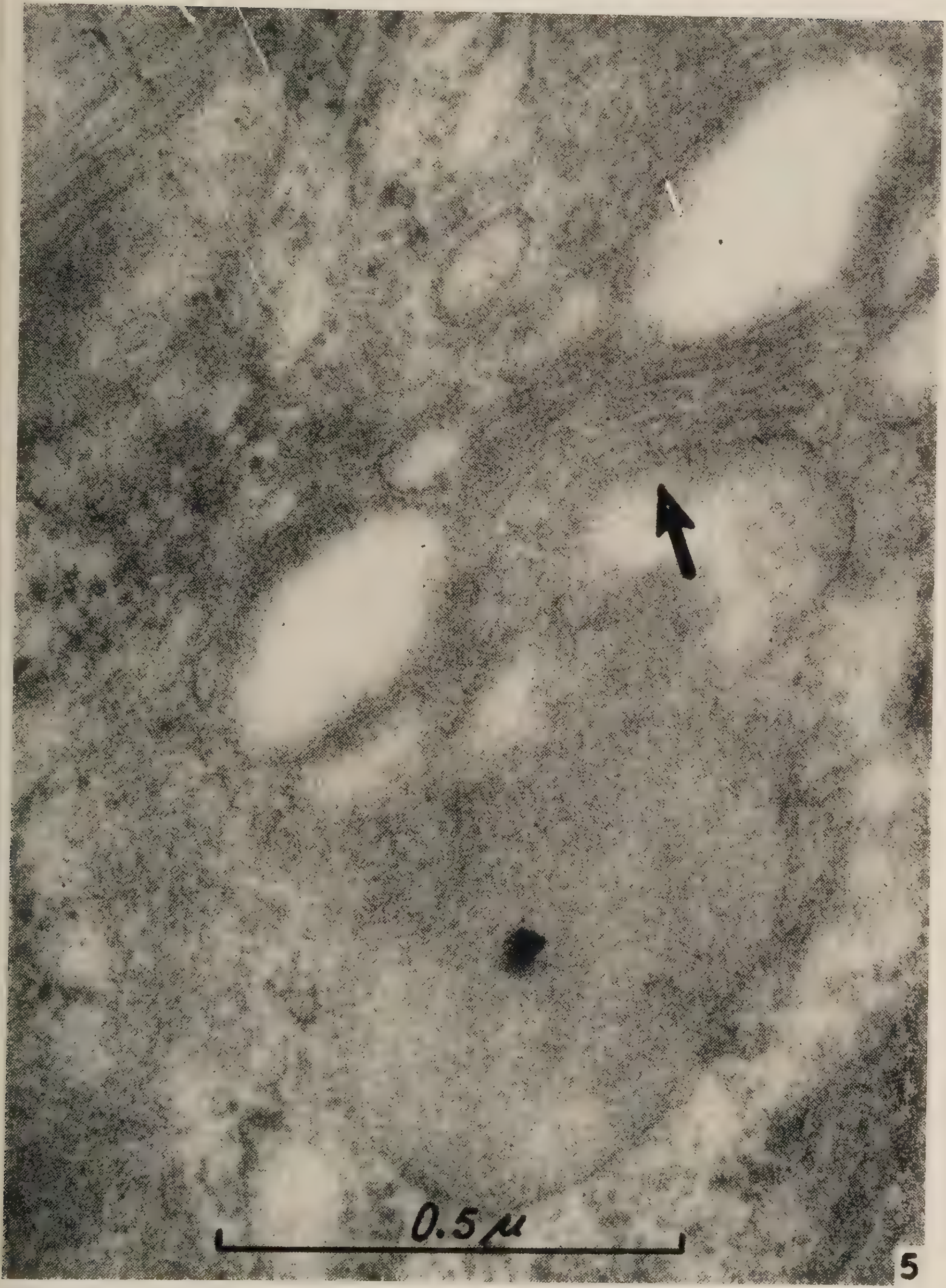

Fig. 5. Golgi membranes presumably split into rows of elongated bodies. In the lower part of the picture a granule diffusely delimited towards the zone of Golgi membrans. Magnification: 126,000 ×.

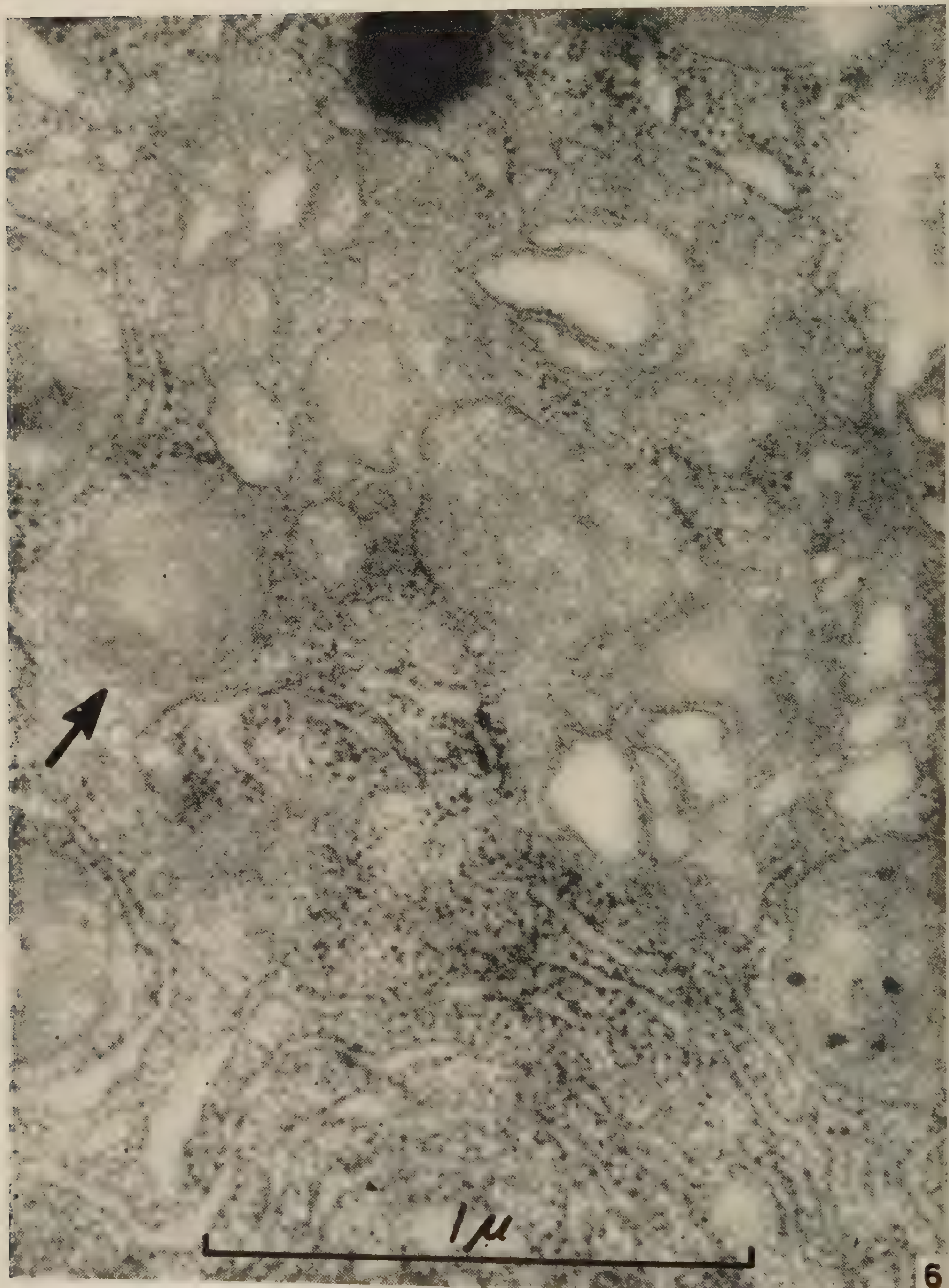

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Fig. 6. Golgi zone with a great variety of Golgi granules embedded in the ground substance. At the arrow a bigger granule associated with a cluster of small granules. Magnification: 74,000 ×.

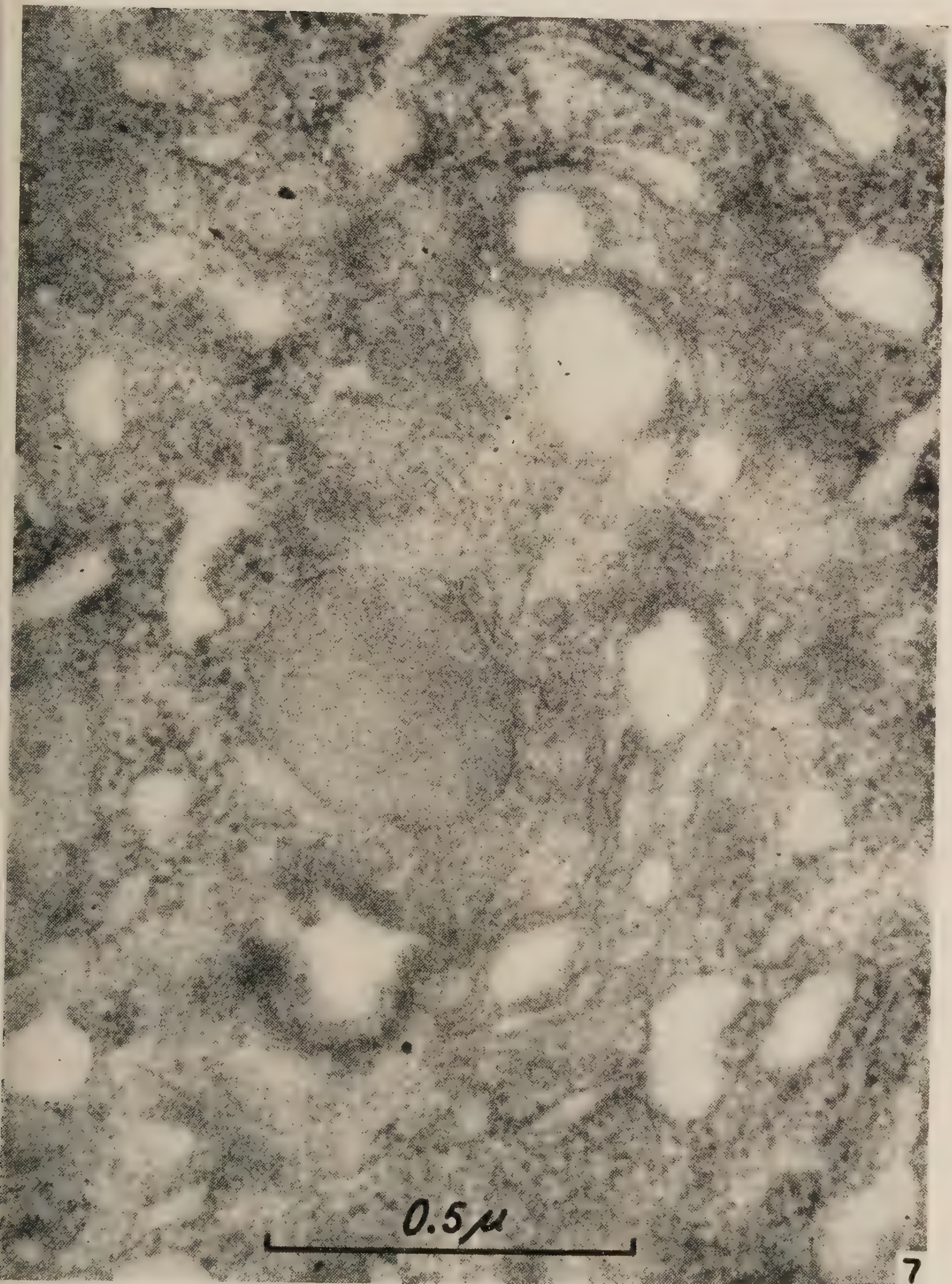

Fig. 7. Golgi zone with many Golgi granules. The big granule in the center is similar to a zymogen granule except for the irregular outlines. Magnification: 99,000 ×.

The granules of the Golgi zone as they appear in the pictures give the impression to represent a whole series of transmission stages from the most minute granules to the well defined zymogen granules with their characteristic rounded form and smooth sharply defined surface. There are all stages regarding size as well as opacity.

The relations of the Golgi apparatus to the mitochondria are not particularly spectacular. Mitochondria are found with intimate topographic relationship to (Figs. 2 and 4) and even within the Golgi zone but the number of mitochondria found here is not conspicuous. No indications of any special accumulation of mitochondria at the Golgi zone has thus been observed. The mitochondria topographically related to the Golgi zone show no modifications as to their structure when compared to mitochondria in other parts of the cell.

DISCUSSION

The structure of the Golgi apparatus as revealed by light microscopy has been interpreted in a rather controversial way. It has been described as a network or as vacuoles surrounded by lipid material (1) as an osmiophobic internum representing a granule surrounded by an externum which is osmiophilic (7), as a twisted intracytoplasmic canal filled with fluid (11) and so on.

The extreme variability of the appearance has been explained by the constantly changing form of the apparatus (2) and as a criterion on its great functional significance. This conclusion seems to have been more attractive than the considering of the variability as reflecting the lack of reproducibility of the methods used for visualizing the Golgi apparatus.

In contrast to the experiences from light microscopy the electron microscopy has revealed the Golgi apparatus as a region of the cytoplasm which appears constantly and is organized in a uniform way. The outer form of this region shows very little geometric regularity but the inner organization appears basically constant in all cells examined. At the stage the present analysis has reached, the following elementary components of the Golgi apparatus have been observed:

1. The Golgi ground substance.
2. The Golgi membranes, arranged in pairs with intercalated vacuolar spaces bounded by the Golgi membranes.
3. The Golgi granules of varying form, size and opacity.

Except for the vacuolar spaces and the bigger granules the dimensions of these components are too minute to make them accessible for light microscopic observations.

The cytoplasmic "strands" described by Dalton and Felix (4) presumably correspond to aggregations of the Golgi membranes and Golgi ground substance as described here.

The observation described in their recent report (5) at the EMSA meeting that the vacuoles were bounded by lamellae similar to the lamellae of the basophilic substance has not been confirmed by the present analysis.

The mutual relations between the Golgi elements and other cytoplasmic structures may be described from the point of view of topographic relationships, at a macromolecular scale. The opaque particles of the cytoplasmic membranes adjacent to the Golgi region show intimate topographic relations to the Golgi ground substance and free such particles may perhaps be present in the outer zone of the Golgi ground substance. Further studies are, however, needed to prove if any opaque particles enter the Golgi region.

The most striking relationship observed is that between the Golgi membranes, the Golgi granules and the zymogen granules. There are all transition stages observed between on the one hand bodies with a pronounced elongated form and with the most direct topographic relations to the Golgi membranes, and Golgi granules embedded in the ground substance on the other. The impression when observing these pictures is that they show snapshots of a conversion of membranes into granules or vice versa. The small granules seem to coalesce to bigger granules which gradually gain the size, form and opacity of the zymogen granules (see for instance Fig. 7).

However, the real relations and genesis of the Golgi and the zymogen granules cannot be established only from topographic relations and apparent morphologic similarities revealed electron optically and assumed to represent a series of stages during a continuous development of granules. In this investigation there has been no control whatsoever of the time relationships, this being definitely necessary for any sound conclusions. Pilocarpine injections do not seem to give useful experimental conditions for this problem. No striking variation of the structure of the Golgi apparatus as due to a pilocarpine effect has been observed in spite of a study of cells obviously in different states of activity as judged from their content of zymogen granules. The great variability reported in light microscopy, it is suspected, might be due to the lack of a perfect technique for visualizing the Golgi apparatus. The variations with cell activity might well be explained by different conditions for staining due to varying interference between adjacent granular structures.

As to the functional significance of the Golgi apparatus we have to wait until properly designed experiments in combination with electron microscopy have been performed. Judging from topographic relationships, which represents a basic principle in morphology, there is nothing pointing against the assumption that the Golgi apparatus is a site for condensation of the secretory products, formed in other regions of the cytoplasm (8, 9). Such an assumption would leave an important task to the cytoplasmic membranes as structures for synthesis.

The electron microscope does not give any direct information regarding the chemical composition of the structures observed. It seems however justified to point out that no drastic reduction of osmium tetroxide has taken place in the Golgi apparatus when fixed in a fresh state in buffered osmium-tetroxide solution. The Golgi apparatus does not appear as more osmiophilic than other regions of the cytoplasm.

No indications of a specially intimate relation between the mitochondria and the Golgi apparatus have been found and no indications of a transformation of mitochondria material into Golgi material according to Hirsch (7) have been observed during this study.

The system of membrane pairs of the Golgi apparatus might well represent a structural principle adapted for enzymatic reaction as might be assumed to be the case regarding the mitochondria membranes.

A structure of the Golgi apparatus similar to the one described here has been observed in the proximal convoluted tubules of the mouse kidney (Rhodin, (10)) and in the epithelial cells and goblet cells of the mouse duodenum (Zetterquist, unpublished).

The high degree of organization of the Golgi apparatus as revealed by the electron microscope seems to indicate that we are dealing with a preformed structure. This organization makes the Golgi apparatus stand out sharply differentiated from the rest of the excretory cell cytoplasm. The differences are so prominent that the structure of the Golgi zones can hardly be explained as a result of local desorganization of this cytoplasm or of the zymogen granules due to postmortal changes or fixation The effects of postmortal factors have been studied (13) and do not result in structures similar to those described for the Golgi apparatus.

On the other hand fixation might well affect the morphology of the Golgi apparatus in producing a swelling of the spaces between the Golgi membranes and consequently produce or enlarge the vacuolar spaces observed. In the embedded material very little material except for the embedding medium is observed within these vacuoles. The reason might be a dissolving of the content during the fixation and embedding as well as an artificial swelling.

SUMMARY

The ultrastructure of the Golgi apparatus has been studied on the exocrine cells of the mouse pancreas under normal conditions of feeding of the experimental animals, after 24 hours of starvation, and after injections of pilocarpine hydrochloride. The electron microscopic analysis has been performed on ultrathin sections of the tissue fixed in buffered, isoton osmium tetroxide solution at +2° C. The best resolution obtained in the series of pictures on which this analysis is based has been better than 30 Å.

The Golgi apparatus is a regularly occurring cell structure located to the distal part of the cells. It represents regions of the cytoplasm which are sharply outlined but the outer form of these regions is fairly irregular.

The internal structure of the Golgi apparatus is rather characteristic. Three elementary components are distinguished.

1. Golgi membranes, arranged in pairs and with 2–5 membrane pairs closely packed except for some intercalated vacuolar spaces.

2. Golgi ground substance, a homogeneous finely granulated or reticulated material.

3. Golgi granules of varying form, size and opacity.

The Golgi membranes and granules are embedded in the Golgi ground substance.

Topographically most intimate relations have been established between the Golgi membranes, the Golgi granules and the zymogen granules.

The Golgi apparatus appears as a structure which is very constant as to its principal organization even when considering cells at various states of activity.

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EXPERIMENTS ON AN ASCITES HEPATOMA

I. ENZYMATIC DIGESTION AND ALKALINE DEGRADATION OF THE CEMENTING SUBSTANCE AND SEPARATION OF CELLS, IN TUMOR ISLANDS

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The production of an ascitic form of rat hepatoma, the cells of which are arranged in aggregates, has been reported by Yoshida and his co-workers (8, 6). Whereas the cells of a typical experimental ascites tumor proliferate free in the peritoneal cavity, those of the ascites hepatoma are associated to form clumps or "islands" (Fig. 1). These structures vary in size from pairs to clusters comprised of one hundred or more cells. As a consequence of the peculiar morphology of this ascites hepatoma, two fundamental problems have been raised. First, what is the nature of the cementing substance which maintains the structural integrity of the hepatoma island; and second, is the association of these tumor cells to form islands a necessary requisite to their continued growth and proliferation in the peritoneal cavity.

Satisfactory answers to both problems depend primarily upon obtaining an effective separation of the constituent tumor cells. In this study, cells of the ascites hepatoma island have been successfully separated by two methods: enzymatic digestion of the intercellular matrix with the aid of trypsin; or, employment of enzyme-free alkaline solutions at pH 9.0 or above. The behavior of tumor cells separated by these techniques and then transplanted into new hosts, is now under investigation.

MATERIAL AND METHODS

Each enzyme[2] solution, except where otherwise indicated, was prepared by dissolving 10 mg of the dry powder in 10 ml of 0.1 M phosphate buffer. Many of

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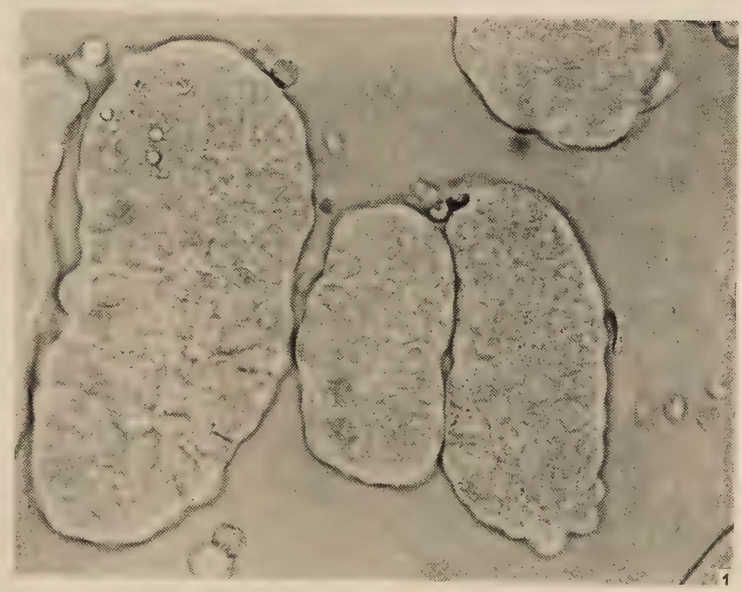
[1] Post-doctorate research fellow: National Institutes of Health, U.S. Department of Health, Education and Welfare.

[2] Enzyme preparations were obtained from the following commercial sources: trypsin (crystalline) and chymotrypsin (crystalline) from Wahl-Henius Inst., Chicago, Ill.: trypsin (2 × crystallized, 50 per cent $MgSO_4$), chymotrypsin (crystalline, salt-free from EtOH), hyaluronidase, papain, diastase (animal), diastase (malt), lipase (steapsin), amylase (alpha), and amylase (beta) from Nutritional Biochemical Corp., Cleveland, Ohio: trypsin (crude, 1 : 250) from Difco Labs. Inc., Detroit, Mich.; hyaluronidase from Schering Corp., Bloomfield, N. J.; hyaluronidase (alidase) from Searle and Co., Chicago, Ill.; Polidase-S from Schwarz Labs., Inc., N.Y.; papain, erepsin and emulsin (amygdalin) from Delta Chemical Works, N.Y.: and ribonuclease (crystalline), and lysozyme (crystalline) from Armour Labs., Chicago, Ill.



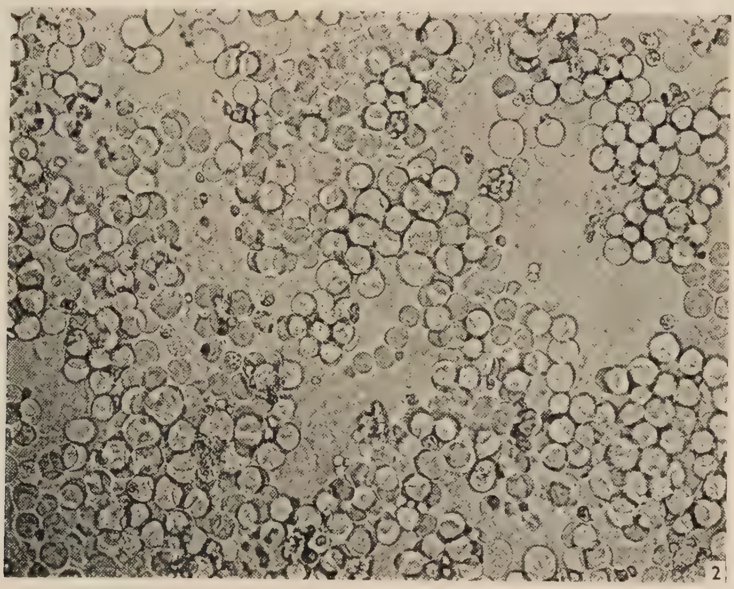
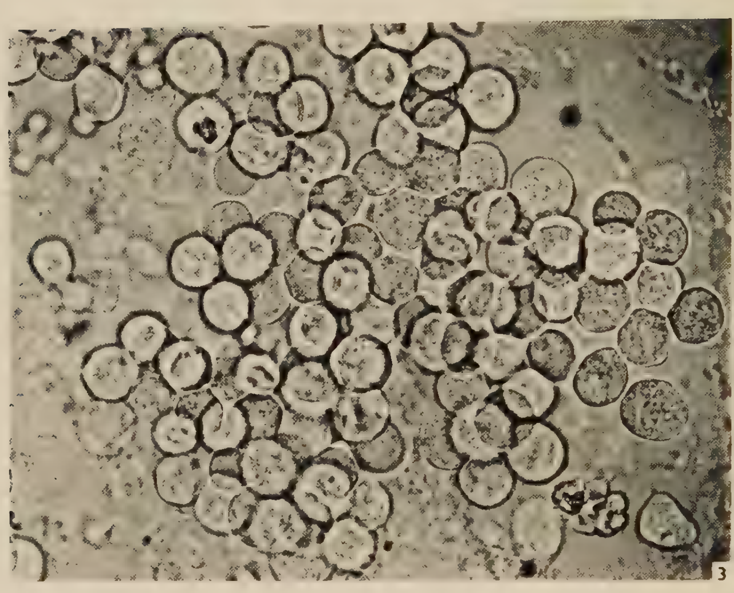


Fig. 1. Untreated cell islands of the rat ascites hepatoma. Magnification 270 ×.
Fig. 2. General view of coagulum containing free tumor cells and unseparated cell aggregates following tryptic digestion. Magnification 270 ×.



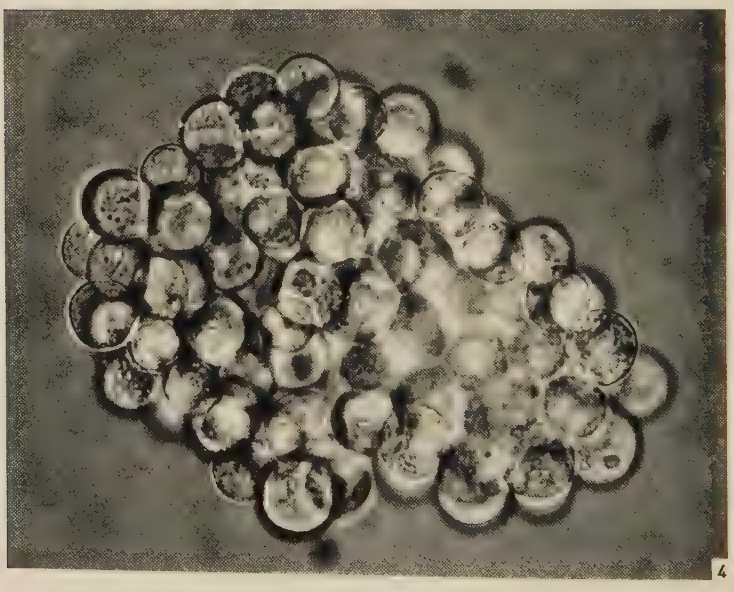


Fig. 3. Separation of tumor cells from hepatoma islands following incubation in 0.1 per cent trypsin for 1 hr. at 37° C. Phosphate buffer: pH 7.3–8.0. Magnification 600 ×.

Fig. 4. Partial separation of hepatoma island following incubation in 0.1 M phosphate buffer (pH 7.0–8.0) for 1 hr. at 37° C. Magnification 700 ×.

these enzymes possessed acid pH optima. However, preliminary experiments on the effect of isotonic phosphate buffer at various pH values indicated that tumor islands were damaged below pH 6.4. Therefore, each preparation listed in Table I was tested at 4 different values all within the range pH 6.4 to 8.0. Crude enzyme solutions were filtered before use and papain solutions were prepared in 0.1 M phosphate buffer containing 0.02 M glutathione as an activator.

The concentration and composition of stock solutions used for the buffer mixtures were as follows: phosphate buffer (0.1 M KH_2PO_4 and 0.1 M Na_2HPO_4); borate buffer (0.2 M H_3BO_3 in 0.1 N NaOH and 0.1 N HCl); veronal buffer (0.1 M sodium diethylbarbiturate and 0.1 N HCl); glycine buffer (0.1 M glycine, 0.1 M NaCl and 0.1 N NaOH); and carbonate-bicarbonate-CO_2 buffer (0.1 M Na_2CO_3 and 0.1 M $NaHCO_3$).

The sources of tumor material were two similar strains of ascites hepatoma, originally induced in a non-inbred stock of Japanese rats by feeding dimethylaminoazobenzene (butter yellow) (8). These tumors were: ascites hepatoma 1 (AH, 1, No. 7974) and ascites hepatoma 2 (AH 2, No. 130). In the present experiments, both tumors were transplanted intraperitoneally into two strains of American rats (M 520 and M 520 × AC) as well as into a Japanese strain. No morphological differences have been observed between ascites hepatoma 1 and 2 carried in these three strains of rats.

Typically, a small quantity of ascitic fluid containing hepatoma islands 3 to 10 days after transplantation was withdrawn from the peritoneal cavity by means of a fine, sterile glass pipette (drawn from 4 mm outside diameter glass tubing). Approximately 0.25 ml of ascitic fluid containing tumor islands was then added to 10 ml of the appropriate solution and incubated for 1 hour at 37° C. The presence of a mucous-like coagulum invariably indicated some form of separation. This coagulum was then microscopically examined for the presence of free tumor cells.

RESULTS

Table I summarizes the results of the action of crude and crystalline enzymes on the cementing substance in islands of the ascites hepatoma. Three trypsin preparations of various degrees of purity and two preparations of crystalline chymotrypsin were capable of effecting a complete separation of tumor cells in many hepatoma islands (Figs. 2 and 3). In addition, Polidase-S, animal diastase and alpha amylase were similarly effective at pH 7.3 to 8.0. Since the pH optimum for animal diastase and alpha amylase is generally considered to be acidic, positive results at a more alkaline pH may have been due to contamination with a proteolytic enzyme. The remaining enzymes tested, including three *preparations of hyaluronidase*, failed to yield cell separation.

The disaggregation of hepatoma islands in solutions of trypsin or chymotrypsin appeared to proceed in two phases. First, digestion of the intercellular

TABLE I

Enzymatic digestion of the cementing substance in islands of an ascites hepatoma.

Enzyme*	Presence of free tumor cells and formation of coagulum (pH 7.3–8.0)
Trypsin (crystalline)	+
Trypsin (2 × crystallized, 50 per cent $MgSO_4$)	+
Trypsin (crude, 1:250)	+
Chymotrypsin (crystalline, Wahl-Henius)	+
Chymotrypsin (crystalline, salt-free from EtOH, Nutritional)	+
Hyaluronidase (Nutritional)	−
Hyaluronidase (Schering)	−
Hyaluronidase (Alidase, Searle, 125 viscosity units per ml)	−
Polidase-S (crude enzyme mixture)	+
Papain (Delta)**	−
Papain (Nutritional)**	−
Diastase (animal)	+
Diastase (malt)	−
Lipase (steapsin)	−
Ribonuclease (crystalline)	−
Amylase (alpha)	+
Amylase (beta)	−
Erepsin	−
Emulsin (amygdalin)	−
Lysozyme (crystalline)	−

* Each preparation contained approximately 0.25 ml of ascitic fluid and 10 mg of enzyme in 10 ml of 0.1 M phosphate buffer, incubated for 1 hr at 37°C.

** Tested at pH 7.0 only.

matrix and subsequent separation of the constituent cells. Second, agglutination of freed cells and unseparated islands to form a mucous-like coagulum which could be promoted by gentle swirling of the suspension.

The presence of protein or other soluble substances in the ascitic fluid was not a requisite for the separation of tumor cells or the formation of a coagulum. Thus, hepatoma islands, washed free of ascitic fluid by several centrifugations and resuspensions in isotonic phosphate buffer reacted typically when incubated with trypsin.

The separation of cell islands in trypsin apparently was due in part to the presence of phosphate. Thus, partial separation of the cells was observed

TABLE II

Alkaline degradation of the cementing substance and separation of cells in islands of the ascites hepatoma.

Solution	pH	Separation of tumor cells
Isotonic sodium chloride (buffered)	7.0	–
Isotonic sodium chloride (adjusted with 0.1 N NaOH)	10.4	+
Krebs Ringer (buffered)	7.0	–
Krebs Ringer* (adjusted with 0.1 N NaOH)	9.0	+
Borate buffer	7.8	–
	9.0	+
Glycine buffer	8.5	–
	8.9	+
	9.9	+
	10.5	+
Veronal buffer	7.6	–
	9.4	+
Carbonate-bicarbonate-CO_2 buffer**	8.8	+
	9.5	+

* Calcium and phosphate omitted.
** Not tested at a lower pH.

following incubation in isotonic phosphate buffer (pH 7.0 to 8.0) in the absence of trypsin (Fig. 4). The characteristic smooth, uniform surface of the island disappeared permitting many cells to become spherical and, in some cases, semi-detached. However, in no case did incubation in phosphate produce complete separation of tumor cells.

It appears that both phosphate and trypsin were necessary for adequate separation of tumor cells. Indeed, solutions of trypsin dissolved in various other buffers (borate, glycine, veronal or carbonate) were typically ineffective even when incubated for three to four hours. Similarly, no separation of tumor cells was obtained when hepatoma islands were incubated in solutions of trypsin dissolved in isotonic salt solutions (NaCl, $MgSO_4$, KCl and $CaCl_2$) or in Ringer's solution.

Tumor islands of the ascites hepatoma were also effectively disrupted in alkaline solutions. Table II indicates the types of buffers and salt solutions employed. Clearly, these solutions, from pH 7.0 to about pH 9.0, had no visible action on the cementing substance in the tumor islands. However, these same solutions when adjusted to greater alkalinity (approximately pH 9.0 or above) produced a separation equivalent in extent and, in some cases, superior to that of trypsin. In the case of salt solutions adjusted to alkalinity with a few drops of 0.1 N NaOH, the pH returned to neutrality following incubation. Nevertheless, this brief treatment was sufficient to effect positive results. Although adequate separation of cells was generally accomplished at about pH 9.0, this value varied slightly depending upon the nature of the solution employed, and no attempt was made to measure exactly the pH range in which separation was effective.

DISCUSSION

The intercellular cementing substance is generally thought to be composed principally of hyaluronic acid or of some type of polysaccharide, often in combination with protein (3). Evidence in favor of this view is based partly upon the specific action of hyaluronidase. That the protein component of the cement substance may likewise often be responsible for maintaining the integrity of certain cellular systems is indicated in recent studies (4, 7). Thus, for example, Mazia and Jaeger (2) immersed salivary glands of *Drosophila* in 0.1 per cent solutions of crude and crystalline trypsin (in 0.05 M K_2HPO_4) and observed an almost complete digestion of the connective tissue, so that ''individual salivary gland cells tended to separate on handling''. More recently, Kaufmann (1) using preparations of trypsin in phosphate buffer at a pH from 6.0 to 9.0 obtained total separation of salivary gland cells in the 3rd instar larvae of *Drosophila*. Similarly Runnström *et al.* (5) demonstrated digestion of the hyaline layer and separation of blastomeres in the egg of a sea urchin by treatment with trypsin.

Results of the present study are in accord with these experiments. An effective separation of tumor cells occurred through the use of trypsin, chymotrypsin or crude preparations of animal diastase, alpha amylase or Polidase-S which might owe their activity at high pH to a proteolytic contaminant. On the other hand, no effect whatever was obtained with three preparations of hyaluronidase. This does not necessarily indicate the general absence in the cement substance of hyaluronic acid or polysaccharide-like substances. The ineffectiveness of these enzymes may be due to several factors, i.e. failure of the enzyme to penetrate the hepatoma island or, since many types of hyaluronidase exist, failure to meet the requirements with respect to enzyme-substrate specificity.

Since many of the enzymes tested were not crystalline preparations, no exact conclusions are possible concerning the nature of the intercellular substance. It appears however, that the structural stability of the hepatoma island depends primarily upon the presence of protein, in the cement substance.

SUMMARY

1. Various types of hydrolytic enzymes were tested for ability to digest the intercellular cementing substance in islands of two ascites hepatomas. Three preparations of hyaluronidase, as well as other carbohydrases and a lipase, failed to cause separation of the constituent cells.

2. Preparations of crude trypsin, crystalline trypsin and crystalline chymotrypsin, in phosphate buffer at pH 7.3 to 8.0, effectively separated cells in many hepatoma islands. This reaction was usually characterized by the formation of a mucous-like coagulum containing almost all of the freed cells. Similarly, Polidase-S, animal diastase, and alpha amylase were effective at pH 7.3 to 8.0.

3. The action of trypsin was due in part to the presence of phosphate. Thus, incubation of tumor islands in phosphate buffer without enzyme, caused a loosening and partial separation of tumor cells.

4. Various enzyme-free salt solutions possessing no action against the intercellular matrix at pH 7.0 to pH 9.0, were effective when the pH was above 9.0. The separation of tumor cells in alkaline solution was equal to, and often more complete, than in the case of trypsin.

5. These data suggest that the ability of the intercellular cementing substance in maintaining the integrity of the hepatoma island may depend largely upon the presence of protein.

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FRACTIONATION OF TRANSMISSIBLE FOWL LEUCEMIA CELL CYTOPLASM ON SILICA COLUMNS

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Fowl leucemia is transmissible through cell-free extracts of leucemic tissue (2). In an erythroleucotic strain the leucemia inducing activity has been found to be connected with the cytoplasm of tumour cells (3).

In order to study the relationship between the tumour agent and the cytoplasm of the tumour cell a fractionation of cytoplasmic material on silica columns has been undertaken and is described in this paper.

METHODS

Strain of leucemia. An erythrogenic leucemia which has been under passage for some years at this laboratory was used. The disease is readily transmissible and is practically 100 per cent lethal. It is among other things characterized by rapidly increasing numbers of immature leucemic cells in the peripheral blood, hepato- and splenomegali and an extensive accumulation of leucemic elements in various organs especially the spleen and liver.

Strain of fowl. An inbred white Leghorn strain (EDO) in which practically no spontaneous leucemia has been noted was used.

Preparation of the cytoplasmic extract (4). About 2 months old hens with advanced leucemia were used. Their spleens were rapidly excised after sacrificing, decapsulated, cut into pieces, pooled and put into 10 volumes of physiological saline. Through gentle homogenizing in a special glass homogenizer a suspension was obtained. To remove tissue débris the suspension was then filtered through 4-double gauze. After these procedures the suspension largely consisted of free cells. About 40 per cent of these were leucemia cells. After having been washed 3 times in 10 volumes of physiological saline in the refrigerated centrifuge the suspension was subjected to 5 minutes treatment in a homogenizer of the Potter-Elvehjem type. After this treatment many cell-membranes were found broken and cytoplasmic material was released. The red blood cells were found to be considerably more resistant than the leucemic cells. See Table I and Fig. 1.

Unbroken cells and nuclei were then separated from the rest through centrifugation at 3,000 × g for 15 minutes. The clear supernatant in the following called the "cytoplasmic extract" was used for the adsorption and elution studies.

Methods of adsorption and elution. Vertical refrigerated cylindrical columns 133 $mm^2 \times 40$ mm of silica in the form of diatomaceous earth (trade name Celite)

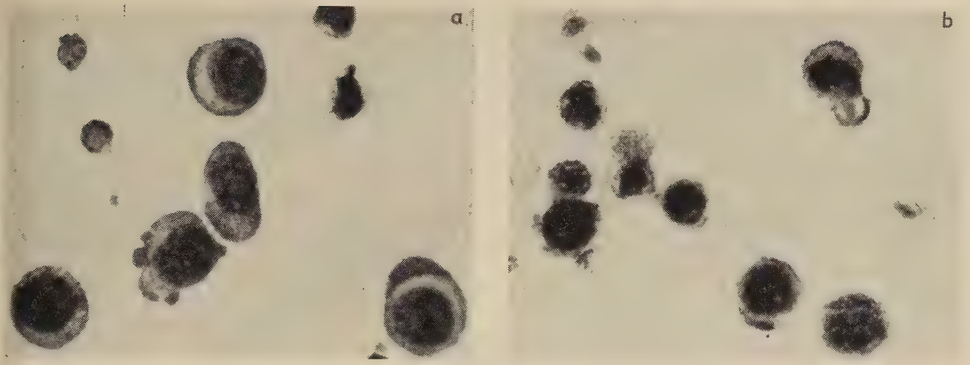

Fig. 1. Appearance of leucemic cells before (A) and after (B) homogenization.

TABLE I

	Undamaged leucemic cells per cent	Leucemic cell "nuclei" per cent	Red blood cells per cent[1]	Lymfocytes reticulum cells etc. per cent	Number of cell nuclei/mm³ (with or without cytoplasm)[2]
Before homogenization	36.9 ± 3.1 $n=10$	1.1 ± 0.5 $n=10$	29.6 ± 3.2 $n=10$	32.4 ± 3.2 $n=10$	376,000 ± 4,000 $n=4$
After homogenization	7.3 ± 1.2 $n=10$	34.7 ± 3.0 $n=10$	30.9 ± 2.4 $n=10$	27.6 ± 1.6 $n=10$	378,000 ± 13,000 $n=4$

Total number of cell nuclei and differential counts of suspensions before and after homogenization. The differential counts were made on smears stained with May-Grünwald-Giemsa. A total of 1,000 cells were counted in each case.

were prepared. The columns were washed with abundant physiological saline before the cytoplasmic extract was applied. The columns were eluted in a nitrogen atmosphere with physiological saline and distilled water under a pressure gradient. For details see Tables II and III.

Assay of leucemia inducing activity. A biological titration method was used with 8 days old chicken as test animals. For details and discussion of the titration method see Fagraeus and Thorell (3). The fraction in question was stepwise ten-fold diluted with distilled water and each dilution injected into the wing veins of a group of 5 animals. In experiment one a primary dilution was made in order to get the same nitrogen concentration in the various fractions. The volume injected into each animal was 1 ml in experiment one and 0.5 ml in experiment two. The survival time was noted and in each instance the spleen was weighed and the diagnosis confirmed

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[1] Practically all red blood cells were morphologically intact after homogenization.
[2] Counted in a Buerker counting-chamber.

TABLE II

	Original cytoplasmic extract	Original percolate	1st eluate with phys. saline	2d eluate with phys. saline	1st eluate with distilled water
Volume (ml)	40	40	20	25	40
γ PNAP/ml	22.0	8.0	6.3	1.1	1.6
mg N/ml	0.712	0.430	0.199	0.017	0.016
$\frac{\text{mg PNAP}}{\text{mg N}} \cdot 1{,}000$	30.9	18.6	31.2	64.7	100.0

Assay of leucemia inducing activity[1]

Dilution	mg N/dose	Original cytoplasmic extract	Original percolate	1st eluate with phys. saline	2d eluate with phys. saline	1st eluate with distilled water
	$1.7 \cdot 10^{-2}$	● ● ○ ○ ○	● ○ ○ ○ ○	● ● ● ○ ○	● ● ● ○ ○	● ● ● ○ ○
	$1.7 \cdot 10^{-3}$	● ○ ○ ○ ○	○ ○ ○ ○ ○	● ○ ○ ○ ○	● ● ○ ○ ○	● ● ● ○ ○
	$1.7 \cdot 10^{-4}$	○ ○ ○ ○ ○	○ ○ ○ ○ ○	○ ○ ○ ○ ○	○ ○ ○ ○ ○	○ ○ ○ ○ ○
	$1.7 \cdot 10^{-5}$	○ ○ ○ ○ ○	○ ○ ○ ○ ○	○ ○ ○ ○ ○	○ ○ ○ ○ ○	○ ○ ○ ○ ○

Experiment 1. Elution diagram.

TABLE III

	Original cytoplasmic extract	3rd eluate with phys. sal. S 3	5th eluate with phys. sal. S 5	7th eluate with phys. sal. S 7	2nd eluate with aq. dest. W 2	3rd eluate with aq. dest. W 3	4th eluate with aq. dest. W 4
Volume (ml)	30	5	5	5	6	6	6
mg N/ml	0.99	0.07	0.06	—	0.04	0.10	0.06

Assay of leucemia inducing activity[2]

	Original cytoplasmic extract	S 3	S 5	S 7	W 2	W 3	W 4
Takes	● ○ ○ ○ ○	● ● ● ○ ○	○ ○ ○ ○ ○		● ● ○ ○ ○	● ● ● ● ○	● ● ● ● ○
mg N/dose	$3.5 \cdot 10^{-2}$	$3.5 \cdot 10^{-2}$	$2.9 \cdot 10^{-2}$		$2.0 \cdot 10^{-2}$	$5.0 \cdot 10^{-2}$	$2.8 \cdot 10^{-2}$
Takes	○ ○ ○ ○ ○	○ ○ ○ ○ ○	○ ○ ○ ○ ○		● ○ ○ ○ ○	● ● ○ ○ ○	● ● ○ ○ ○
mg N/dose	$3.5 \cdot 10^{-3}$	$3.5 \cdot 10^{-3}$	$2.9 \cdot 10^{-3}$		$2.0 \cdot 10^{-3}$	$5.0 \cdot 10^{-3}$	$2.8 \cdot 10^{-3}$
Takes	○ ○ ○ ○ ○	○ ○ ○ ○ ○	○ ○ ○ ○ ○		○ ○ ○ ○ ○	● ○ ○ ○ ○	○ ○ ○ ○ ○
mg N/dose	$3.5 \cdot 10^{-4}$	$3.5 \cdot 10^{-4}$	$2.9 \cdot 10^{-4}$		$2.0 \cdot 10^{-4}$	$5.0 \cdot 10^{-4}$	$2.8 \cdot 10^{-4}$

Experiment 2. Elution diagram.

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[1] Each circle represents one animal. The black circles mean leucemic deaths.
[2] Each circle represents one animal. The black circles mean leucemic deaths.

by microscopy of liver sections. The mean survival time was 21 days with 11 and 62 days as extremes.

The time between the start of the experiment and the injection of the material into the test animals did not exceed 48 hours. The temperature of the cytoplasmic extract was never allowed to exceed +4° C.

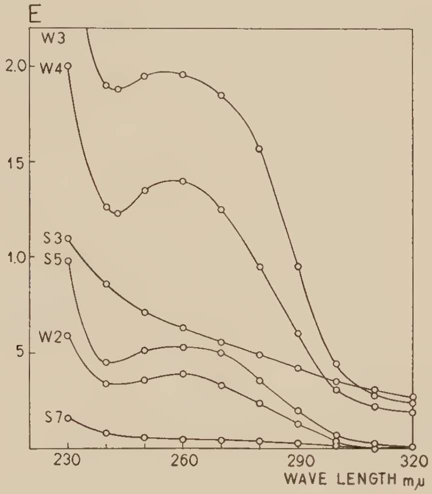

Fig. 2. Ultraviolet absorbtion curves from some of the eluates from experiment two. The curves are labeled according to the designations in Table III.

To minimize any possible systematic error depending on an eventual inactivation of the material during the injection of the test animals, the animals were injected as rapidly as possible and in no systematic order.

Chemical determinations. The nitrogen was determined according to a Kjeldahl semi-micro method.

The PNA determinations were made according to a method described by Brody (1) on trichloracetic acid extracts.

All determinations were run in duplicate. The tabulated values are the means of the duplicate analyses.

RESULTS AND COMMENTS

Two experimental series have been performed. See Tables II and III and Fig. 2.

From Table II can be seen that the ratios $\frac{\text{mg PNAP}}{\text{mg N}} \cdot 1{,}000$ differ from each other in the various fractions. The lowest value was found in the original percolate and the highest value in the eluate with distilled water. A heterogenous distribution of leucemia inducing activity (calculated on a nitrogen basis) was also obtained. The original percolate exhibited the lowest activity and the eluate with distilled water the highest.

No greater loss of the leucemia inducing activity seemed to have taken place.

In experiments of the above mentioned type other factors than specific adsorption phenomena cannot be excluded to play a role. As examples may be mentioned breaking up of particulate matter in distilled water and a non-specific filtration with different rates of propagation for particles of different size. To get a better concentration of the leucemia inducing activity and also to exclude the last mentioned possibility it was decided (experiment two) to continue elution with physiological saline until no more nitrogen was detected and no specific light absorption at 260 mμ was obtained in the eluates and then elute with distilled water. Table III and Fig. 2.

Table III and Fig. 2 show that in the presence of physiological saline part of the cytoplasmic extract was selectively retained on the column and that when elution with distilled water was commenced part of this material was relased.

The last mentioned material was characterized by a high selective ultraviolet absorption at 260 mμ. See Fig. 2.

Although the data from the biological assay of the leucemia inducing activity do not permit a strict quantitative treatment it seems obvious that a 10–100 fold increase of this activity calculated per total nitrogen has been obtained in some of the eluates with distilled water. (W.3 and W.4.) See Table III.

SUMMARY

The cytoplasmic fraction from the erythrogenic leucemia cells has been adsorbed on silica columns. Elution was made with physiological saline and distilled water.

No greater loss of the leucemia inducing activity took place.

A heterogenous distribution of the PNA content was obtained.

A 10–100 fold concentration of the leucemic activity calculated per total nitrogen was obtained in some of the eluates with distilled water.

This investigation has been supported by grants to the laboratory from the Swedish Medical Research Council and the Lady Tata Foundation.

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MITOSIS IN CULTURES OF NEWT TISSUES

III. CLEAVAGE AND CHROMOSOME MOVEMENTS IN ANAPHASE

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The phenomena of the anaphase and telophase of mitosis have been studied in living cells by several observers. Bělař (2) measured the rate of chromosome movement during anaphase in the spermatocytes of the grasshopper *Chorthippus* and Barber (1) made similar observations on the cells of the staminal hairs of the flower *Tradescantia*. Ris (20) found that in secondary spermatocytes and embryonic cells of the aphid *Tamalia*, the chromosomes first moved towards the spindle poles and then the spindle elongated. Ris showed that in general, anaphase movements consisted of one or both of these processes and Hughes and Swann (18), using cultures of embryonic chick fibroblasts, distinguished elongation of the spindle from the movement of chromosomes towards the spindle poles. In the present studies, already reported briefly elsewhere (5), it has been found that anaphase in newt fibroblasts cultivated *in vitro* takes place in two stages corresponding to those reported by Ris for *Tamalia*. Thus the fibroblasts of the newt provide another example of the temporal separation of the two mechanisms of chromosomal movement during anaphase; in these cells there is also a constant time-relation between spindle-elongation and cleavage.

METHODS

Newt fibroblasts were cultivated *in vitro* and photographed alive by the methods previously described (6). The only stained preparations used in the present studies were tissue cultures fixed in Flemming's solution, stained with Flemming's triple stain and mounted whole.

OBSERVATIONS

Changes in cellular shape during mitosis. Cells shortened during late prophase and in metaphase, and became most rounded at the end of metaphase or in early anaphase; after cleavage, which began in anaphase, the daughter cells acquired the irregular and extended form characteristic of interphase (Fig. 1).

Although mitosis was sometimes detectable four hours before metaphase, the shape of the cell altered little before the last half-hour of prophase, when the nucleoli were disappearing most quickly; at this time some cells became rather rounder. In metaphase, however, cells rapidly assumed a more regular outline, achieving a circular optical cross-section in late metaphase or very early anaphase.

While the cell was thus becoming increasingly compact in form, its processes were reduced in any of three ways. Some were completely withdrawn. Others were replaced by one or more very fine threads (less than 0.5 μ in diameter) which extended for the distance covered by the original process and were formed during its withdrawal; these threads, like those in films by Canti (9), appeared to be drawn out when the distal part of a retracting process failed to detach itself completely from the medium or cover slip (A in Fig. 1 b and d). Besides complete withdrawal or replacement by fine threads, a cytoplasmic process, especially the main continuation of a fibroblast, often became reduced in width but not in length; when the rounding of the cell was complete such a process had shrunk to 1–2 μ in diameter and bore many small, round protrusions (B in Fig. 1 d). The protrusions may have represented the accommodation of the surface of the process to a reduced volume; they differed from those on the cell-body (to be described in the fourth communication of this series) in being relatively stable, whereas the latter were continually protruded and withdrawn.

Cleavage of the rounded cell began in anaphase and was completed in telophase. Two types of cleavage were observed. In the first, which occurred in outgrowths of pulmonary epithelium and usually in perichondrial fibro-

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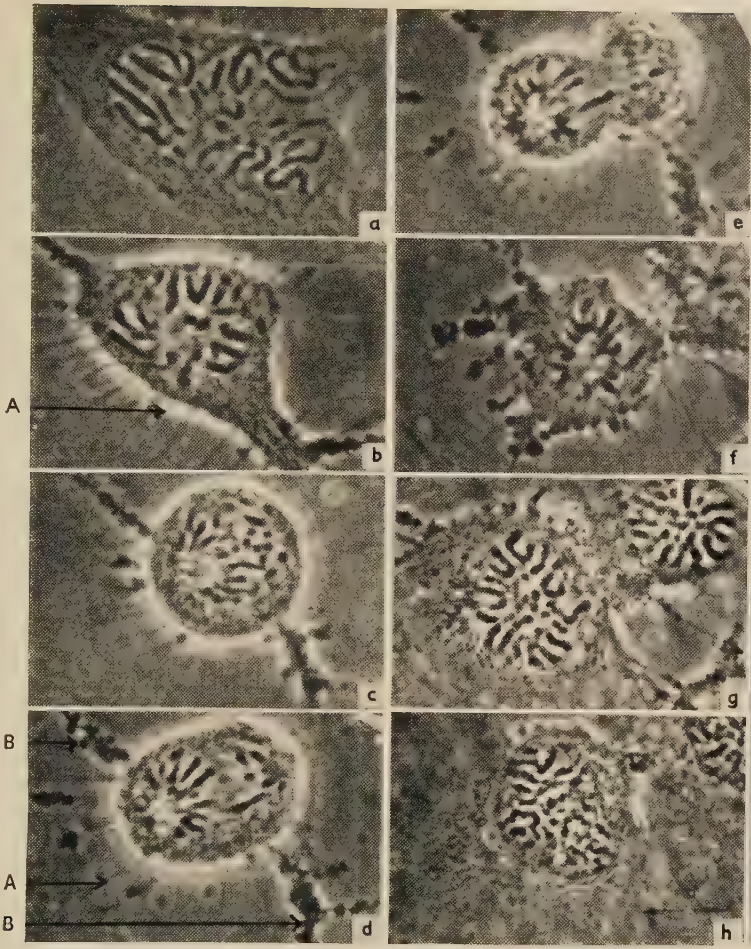
Fig. 1. Living perichondrial fibroblast in mitosis. (a) Early metaphase. The cell is less spread out than it had been in prophase. (b) The cell is more compact and its surface is connected by very fine threads, A, to the region from which it has withdrawn. The prolongations of the cell are becoming narrow and are beginning to bear small projections. (c) The cell is completely rounded and its processes are at their narrowest; anaphase is beginning. (d) Mid-anaphase. The cell has become longer and narrower. A — fine processes connecting the cell to the region from which it has withdrawn. B — narrowed processes bearing rounded protrusions. (e) The narrowing at the equator has led to the formation of a cleavage-furrow. The cleavage is of the direct type, unmodified by the unretracted processes, to which the mitotic axis is not related. (f) A daughter cell with clumping of chromosomes, "bubbles" at the surface of the cell-body and some spreading of the cytoplasm. (g) The nuclear membrane has formed, "bubbling" has ceased and the cell has spread out further. (h) The spreading of the cell has continued, and the cytoplasm extends as a thin sheet away from the direction of the cleavage plane. A few fine strands connect the daughter cells, and the chromosomes are beaded. Phase-contrast. × 900.

Time intervals: (a)–(b), 21 minutes; (b)–(c), 34 min.; (c)–(d), 9 min.; (d)–(e), 7 min.; (e)–(f), 23 min.; (f) (g), 32 min.; (g)–(h), 46 min.

Fig. 2. Living cardiac fibroblast. 3 minutes between photographs (a) and (b). Phase-contrast. × 750. Cleavage by simple furrowing.



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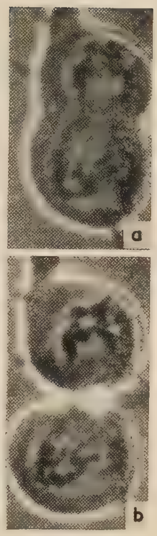

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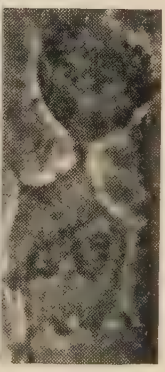

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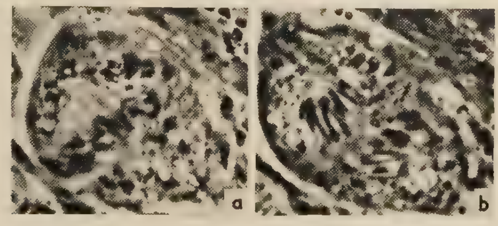

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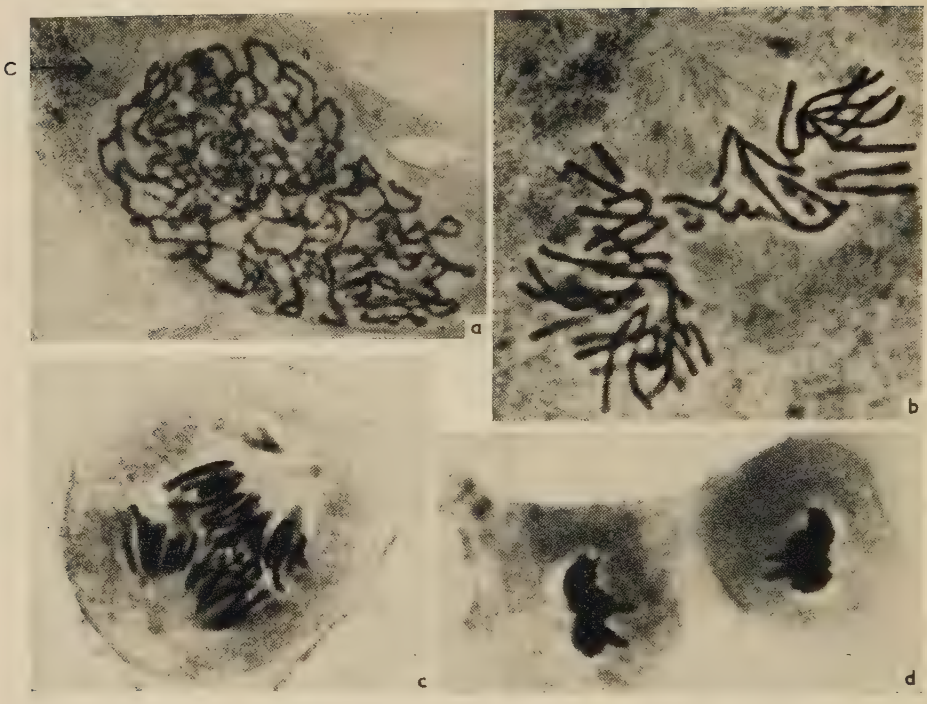
Fig. 3. Living perichondrial fibroblast. Phase-contrast. × 750. Cleavage with a connecting isthmus drawn out by the separating daughter cells.

Fig. 4. Living cardiac fibroblast. $9\frac{1}{2}$ minutes between photographs (a) and (b). Phase-contrast. × 750. There appear to be spindle fibres running from the centromeres of the upper group of chromosomes to the spindle pole.



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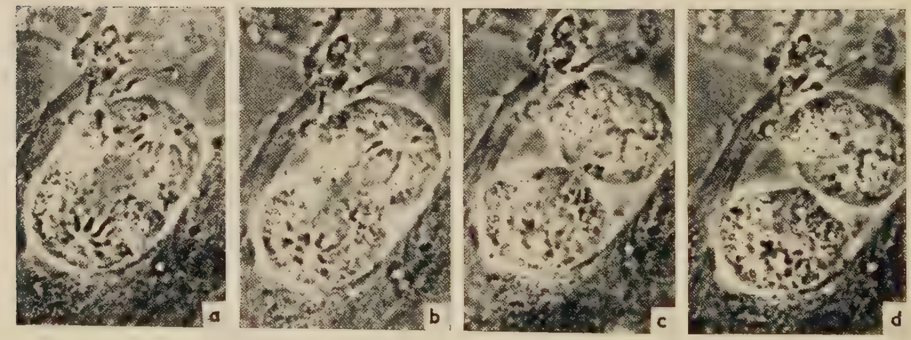

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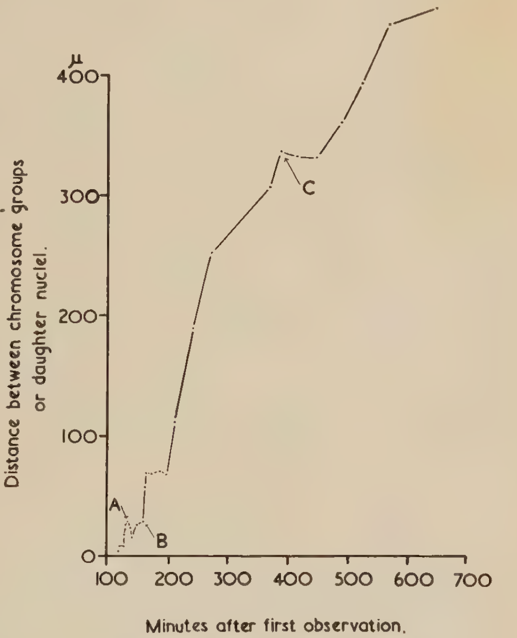
Fig. 5. Four fibroblasts in a heart culture. Flemming's fixative and triple stain. × 1500. (a) Nucleoli lost and nuclear membrane breaking down; C – aster (with centrosome?). (b) Lateral view of metaphase plate, showing spindle. (c) Mid-anaphase; note spindle-fibres converging from the chromosomes to the spindle-poles. (d) Cleavage; no spindle-fibres are present except some short lines from the poleward ends of a few chromosomes.

Fig. 6. Living cardiac fibroblast. Phase-contrast. × 620. (a) Anaphase, with equatorial narrowing. (b) The cleavage furrow is approaching the interchromosomal part of the spindle. (c) The furrow is penetrating the spindle area. (d) The furrow has severed the spindle, the interchromosomal part of which is represented by a clear area in each daughter cell.

Time intervals: (a)–(b), 4 minutes; (b)–(c), 3 min.; (c)–(d), 2 min.

blasts, a furrow incised the cell into two rounded daughter cells (Fig. 2); the general appearance thus resembled cleavage in, e.g. echinoderm eggs, but the newt cells in tissue culture soon lost their smoothly rounded form. In the fibroblasts of heart and lung cultures, cleavage was usually of the second type, in which the cell lengthened rapidly before division was complete and the daughter cells, which were not rounded or only very transiently so, moved apart while still connected by a cytoplasmic isthmus (Fig. 3); they often remained connected by a thread-like bridge after resuming an interphase appearance and moving up to 0.5 mm from each other (Text-fig. 1). In this type of cleavage the parent cell was often shaped like an hour-glass during division.



Textfig. 1. Graph indicating the separation of the daughter cells of a perichondrial fibroblast. A — cleavage-waist becomes less than 5 μ wide. B — Membranes form around daughter nuclei. C — cytoplasmic connection between daughter cells breaks. The daughter cells separated at a maximum speed of 3 μ per minute, and were nearly 0.5 mm. apart 8 hours after the beginning of telophase. After the cytoplasmic isthmus broke, the free ends of the processes so formed did not retract.

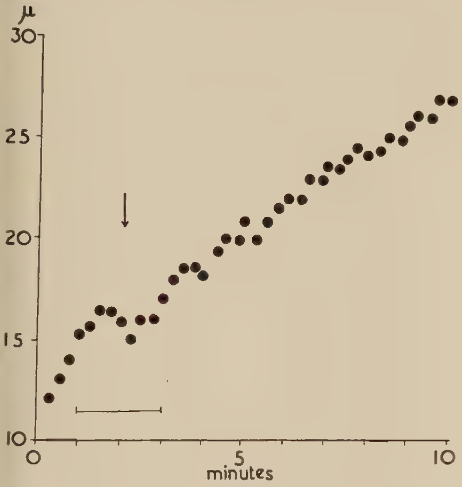
Dr. M. M. Swann and Dr. J. M. Mitchison very kindly put their polarising microscope at my disposal, enabling me to observe the birefringence of dividing fibroblasts in the outgrowth of cultures of newt lung. These mitotic cells had long, unretracted processes of the broader type mentioned above, which were positively birefringent, showing longitudinal orientation. During cleavage there could be seen a line of birefringence from a process attached near one pole of the dividing cell to one near the opposite pole, passing to one side only of the two daughter chromosome groups, and through the connecting neck of cytoplasm between the daughter cells. This line was distinct from the weaker birefringence of the spindle. The cleavages observed with the polarising microscope were of the type in which the connecting cytoplasmic neck was drawn out as the daughter cells moved apart (Fig. 3), and it seems possible that there was tension between the processes, transmitted by the dividing cell. In this connection it was significant that the first direction in which the daughter cells moved was along the line of their unretracted processes; when these were not in line with each other, the daughter cells separated along paths which deviated from a single straight line. By contrast, in cells which underwent direct cleavage (Figs. 1 and 2) there was no orientation of either the mitotic figure or daughter cells by the unretracted processes. The drawing-out of the cytoplasm between the daughter cells may have been due to the effect of tension in unretracted processes being superimposed on the mechanism of cleavage by simple furrowing.

After cleavage the daughter cells assumed the interphase form, becoming flattened in the plane of the outgrowth and again protruding cytoplasmic processes. The very fine threads disappeared as the cell spread back over the areas across which they stretched, while the broader unretracted processes filled out, sometimes becoming as wide as the greatest diameter of the cell body, and their rounded projections disappeared as the spaces between them were obliterated.

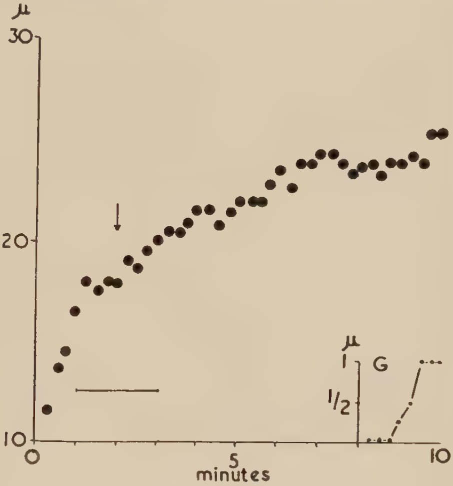
Separation of chromosomes. The separation of the groups of daughter chromosomes in anaphase followed a characteristic course temporally related to the beginning of cleavage.

Measurements were made between opposite centromeres in photographs of living cells viewed laterally, and these were plotted against time. Before the results of measuring centromere-separation are given, certain possible sources of error must be considered. In actual measurement the inaccuracy was probably less than 0.5 μ when photographs were taken with the 1.3 N.A. objective; a resolution of about 0.3 μ was obtainable between chromatids of living metaphase cells, and the total magnification used in studying anaphase movements was ×1,000. An important source of error might arise from movement of the centromeres out of the plane of the photograph, so that the path along which their separation was measured was only the projection of the true path on to a plane. This, however, can hardly have affected the results, since the depth of focus of the objective was small and measurements were always made between centromeres which remained in focus throughout the series of photographs, although the focussing adjustments of the microscope were left untouched.

After preliminary observations at lower magnifications, photographs were taken at quarter-minute intervals with the 2 mm oil-immersion objective. Graphs such as that in Text-fig. 2 were obtained when separation of centromeres was plotted against time. A "step" in the graph is evident; Text-fig. 2 shows that anaphase occurred in two distinct stages with a pause between them, the first stage being shorter and faster than the second. As it was feared that the light necessary for photography at this magnification might damage a cell in metaphase, which was found to be a very light-sensitive period, photography was begun only when the cell, viewed by dim illumination, was seen to have entered anaphase.



Textfig. 2.



Textfig. 3.

Textfig. 2. Graph recording the separation of a pair of centromeres seen near the apparent axis of the mitotic figure in a living fibroblast in anaphase. From photographs taken at quarter-minute intervals with a 2 mm objective, by phase-contrast. The movement of separation falls into two parts separated by a "step". The arrow shows the time when the centromeres reached their greatest degree of crowding together, that is, when they reached their nearest positions to the spindle pole. The horizontal line marks the period when most of the chromosome tips were disengaging from those of the opposite group. (Published by permission of the *Journal of Physiology.*)

Textfig. 3. Graph, shown by round spots, comparable with that of Text-figure 2, but derived from the longest pair of chromosomes in the same mitotic figure. The arrow marks the arrival of the centromeres at their nearest positions to the spindle-poles. The horizontal line marks the period when *most* of the trailing chromosome tips were disengaging across the equatorial plane. The graph G shows the separation of the tips of the pair of chromosomes used for the principal graph.

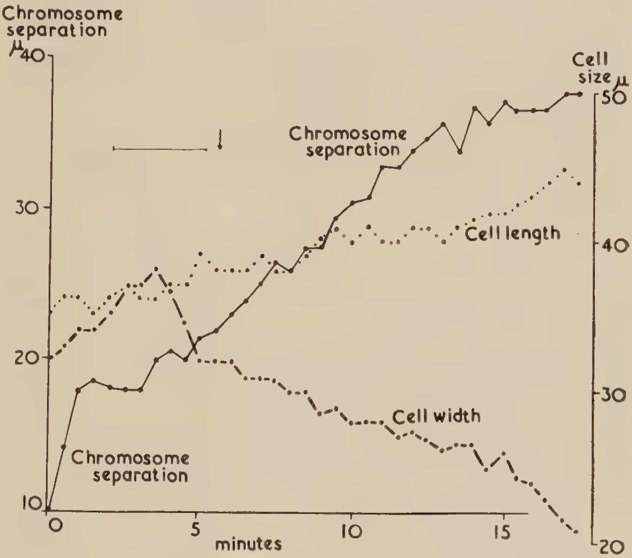
The first part of anaphase movement took place with a speed of separation of 3–8 µ per minute, and the second at about a quarter of this rate. The interval between the movements lasted 1–3 minutes, during which time the chromosomes either were motionless or approached one another slightly. Graphs were drawn for the movements of 13 pairs of chromosomes in ten fibroblasts and one epithelial cell. Nine pairs of chromosomes in seven fibroblasts (with both types of cleavage) separated in the manner shown in Text-fig. 2. The remaining four pairs of chromosomes yielded results that were equivocal, either because a true lateral view of the cell was not obtained, or because some non-chromosomal material partially obstructed the view.

In order to see the relation of the anaphase movements to cytoplasmic changes, it was necessary to examine how the former were related to the spindle. The poles of the spindle could be located in phase-contrast photographs of living cells in three ways. First, the spindle was often distinguishable as an area, well-defined near the poles, free of mitochondria and fat globules; second, the poles could be identified as the points towards which chromosomes converged, and third, it was sometimes possible to locate spindle poles directly as the points of convergence of streaks corresponding in position to chromosomal spindle fibres (Textfig. 4); these streaks will be considered in the description of the spindle. Thus the distance between a centromere and the spindle-pole towards which it was moving, could be measured, and by plotting this distance against time, the time required for the centromere to arrive at its position of nearest approach to the spindle-pole could be determined. This time could also be ascertained by direct inspection of a series of photographs, since centromeres approaching the poles became increasingly crowded together and the line formed by them was more and more sharply curved; the crowding and curvature ceased to increase when the centromeres reached their nearest positions to the poles.

In Text-fig. 2 the arrow indicates the time at which the centromeres completed their approach to the spindle-poles, and shows that the break in the continuity of anaphase-separation occurs when the movement of the chromosomes along the spindle stops.

While it was always found that movement along the spindle ended at the time of the inflexion in the graph of chromosome separation, before assuming the probability of a causal relation between the two events it was necessary to see whether the inflection should have been associated rather with another event occurring at the same time as the cessation of the approach to the spindle poles. In Text-fig. 2 the horizontal line below the graph indicates the time at which the tips of the chromosomes disengaged at the mid-plane; because of the differences in length of the chromosomes, this line indicates the separation of most but not all of them. The problem of whether to associate the pause in anaphase-separation with the stopping of movement along the spindle or with the disengagement of the chromosome tips, could be largely solved with reference to the cell from which the graph of Text-fig. 2 was derived.

In each anaphase group of this cell, there was one chromosome which was longer than the others and which had not lost contact with its sister chromosome even after the remainder of each group had moved sufficiently to leave a space between them at the equator; the tips of the pair of long chromosomes therefore disengaged after the movement along the spindle was complete, the latter event having taken place, however, when most of the chromosomes were disengaging. The principal graph in Text-fig. 3 indicates the separation of the centromeres of the pair of long chromosomes and Graph G the separation of their tips. It can be seen from Text-fig. 3, that the inflection in the graph of centromere-separation does not necessarily occur at the same time as the separation of the tips of the same pair of chromosomes. Text-fig. 3 strongly suggests that the pause in anaphase-separation and the disengagement of most of the chromosome tips happen at the same time only because the length of most of the chromosomes is such as to make the two events coincide. The inflection in the graph of centromere-separation should be associated therefore with the cessation of movement along the spindle, rather than with the disengagement of the trailing ends of the chromosomes.



Textfig. 4. Graphs showing the separation of a pair of centromeres, the length of the cell and its width. Based on photographs taken by phase contrast microscopy at half-minute intervals with the 2 mm objective. The arrow indicates a time after which there was no further movement of the chromosomes towards the spindle-poles. The horizontal line marks the time when most chromosome tips were disengaging at the equatorial plane. (Published by permission of the *Journal of Physiology*.)

The position may now be stated thus: anaphase separation in these cells took place in two stages, a rapid movement along the spindle itself and a longer and slower movement, following the first and separated from it by a pause. The nature of the second movement will become clearer when anaphase separation is correlated with other processes which occur simultaneously in the cell. Analysis of changes recorded in photographs taken with the dry 4 mm objective, showed that a considerable diminution in width and some increase in length of the cell begins at about the time of the change of movement of the separating centromeres. Text-fig. 4 enables the time-relations to be analysed more accurately as it is based on photographs taken at half-minute intervals with a 2 mm oil-immersion objective; the arrow and the horizontal line have the same meaning as in Text-figs. 2 and 3 save that, owing to difficulty in analysing the photographs, the arrow indicates not the exact time at which movement along the spindle ceased, but a time by which it had already done so. The curve of chromosome-separation is of the same type as those already discussed. It is evident from Text-fig. 4 that the second anaphase movement began as the equatorial width of the cell started to decrease rapidly, whereas cell-length increased slowly at first, suggesting that the first active process in cleavage is narrowing rather than lengthening. Between the fifth and fifteenth minutes of observation, the length of the cell increased by 7 μ and the separation of the pair of centromeres by 30 μ; from this it follows that the second part of anaphase separation is not a consequence of general cytoplasmic elongation, but is associated with a lengthening of the interchromosomal part of the spindle, which at this stage is usually distinguishable in phase-contrast photographs of the living cell as a region of cytoplasm free of organelles and inclusions.

It is concluded that the separation of centromeres in fibroblasts in cultures of newt tissues takes place in two stages, a movement along the spindle followed by the elongation of the interchromosomal part of the spindle, the second movement beginning at the same time as the equatorial narrowing that leads to cleavage.

The spindle. In fixed preparations the spindle was not detected before the disappearance of the nuclear membrane, and it ceased to be demonstrable after the clumping of the chromosomes (*i.e.* when they had completed their second anaphase movement) (Fig. 5).

With the polarising microscope it was possible to examine the birefringence of the spindle in living newt cells; it was found to be low compared with that of, *e.g.*, a chick fibroblast. The weakness of the birefringence may have been due to a low degree of orientation or to the opposing effects of form and intrinsic birefringence.

By phase-contrast microscopy the spindle in the living cell, as already mentioned, was demarcated by excluded mitochondria and fat globules, and lines corresponding to chromosomal spindle fibres could sometimes be seen (Fig. 4); this agrees with the appearance in living blastomeres of the mite, as described by Cooper (11). Although the interchromosomal part of the spindle was often constricted by the cleavage furrow, the latter could sometimes be seen to cut through the spindle (Fig. 6).

Locomotion. During mitosis a cell sometimes wandered bodily while the mitotic changes were proceeding within it. In metaphase a rounded cell might migrate as much as 100 μ, and one cell was seen to develop a tail like that of an amoeba and move at 5–6 μ per minute until the beginning of a normal anaphase. In telophase, daughter cells moved apart, and this separation continued after the cells were detached from each other (Text-fig. 1).

DISCUSSION

The results just presented allow the temporal correlation of nuclear and cytoplasmic changes during the mitosis of newt fibroblasts cultivated *in vitro*. Cleavage began with the second anaphase-movement, which was due to elongation of the interchromosomal part of the spindle, and was never seen to begin during the first period of anaphase, when the chromosomes were moving along the spindle.

Barber (1) found that the rate of movement of chromosomes during anaphase in *Tradescantia* was 0.5 μ per minute in the cells of the staminal hairs, as compared with 1–8 μ per minute in newt cells in the present work. Ris (20) showed that anaphase movements in living cells of the aphid *Tamalia*, proceeded in two stages, comparable with those described here. In chick (18) and mouse cells (15) anaphase movements showed no discontinuity, although Hughes and Swann, by measuring the length of the birefringent spindle region, were able to distinguish elongation of the spindle from movement of the chromsomoes along it. The difference between the smooth curves for anaphase movements in chick or mouse cells and the inflected curves for some newt or aphid cells depends on the temporal relations of the two mechanisms; if movement along the spindle and lengthening of the spindle itself overlap in time, then a graph of anaphase movements may not show an inflection such as that present when one process ends before the other begins. Hughes and Preston (17) are the only previous workers to have measured rates of chromosome-separation in amphibian cells. They do not record anaphase-movement taking place in two stages, but their graphs, derived from cells of the crested newt *Triturus cristatus,* begin when the centromeres are already 10 μ apart, so that it is impossible to compare their results with those given here; in the present studies, however, it has been found that the first movement continues to a rather greater separation than 10 μ, and thus there may be some genuine divergence between the two sets of data, perhaps because the types of cell chosen for study were not the same.

Bělař (2) found that in the first third of anaphase in the second spermatocyte division of the grasshopper *Chorthippus* daughter chromosomes remained parallel while separating. This seems to correspond, not to the first movement of anaphase in newt fibroblasts, but to the smaller parallel separation of the limbs of newt chromosomes in late metaphase, before division of the centromeres.

Of the several theories proposed to explain anaphase movements, none is entirely satisfactory, as is shown by the discussion in Schrader's monograph (21), and many of the difficulties encountered may have arisen through a too rigid insistence on a single immediate cause. In the present series of observations the first movement of anaphase was at a constant velocity. If this movement were due to repulsion between the chromosomes or attraction of the centrosomes for the centromeres, then a constant velocity would not be expected if the forces acted according to an inverse square law across a region of cytoplasm without variations in physical properties. Traction by the fibres between the chromosomes and spindle poles would not give a constant chromosome-velocity if the fibres were elastic and obeyed Hooke's law, but might perhaps do so if they were actively contractile; Lewis (19) has laid stress on contractile mechanisms in cell division. As anaphase movement has not been timed with frequent observations (quarter- or half-minute intervals) during its first 1–2 minutes in the present experiments, it is not known how the chromosomes move at the onset of separation, but measurements at less frequent intervals suggest that the velocity becomes constant very early. A theory requiring contractile or elastic chromosomal spindle-fibres can explain chromosome movements only if there is some mechanism anchoring the spindle poles in relation to the cell surface.

During the second anaphase-movement, when the chromosomes no longer approach the spindle poles and the interchromosomal part of the spindle elongates, the cell as a whole lengthens only about as much as would be expected from the division of one sphere into two. Darlington (14) has put forward the view that lengthening of the spindle is due to interchromosomal repulsion, and Botta (7), among others, has demonstrated a negative charge on chromosomes. It has also been suggested that the interchromosomal part of the spindle is a solid structure which forces the chromosome groups apart as it lengthens. Thus Bělař (2) found that interchromosomal fibres in grasshopper spermatocytes were deformable, and he called them *Stemmkörper* (prising bodies). Similarly, Berkeley (3) found that when anaphase movements were impeded in the giant amoeba, the interzonal spindle continued to lengthen and buckled. Bonnevie (4) also observed deformation of spindles, and Dan (12) has taken the view that asters are pushed apart by the lengthening spindle.

The striking coincidence of the onset of cleavage with that of the second movement of anaphase has proved a constant feature of mitosis in newt fibroblasts in tissue culture whenever the two stages of anaphase have been distinguishable. The present work does not directly indicate the mechanism of cleavage, but in view of the way in which the furrow appears to cut the spindle, it is unlikely that cleavage can be due to a falling in of the equatorial cell surface because of cellular elongation, as Gray (16) once supposed; cleavage due to elongation would, moreover, be expected to begin when the length of the cell was π times the diameter, but in fact the ratio of length to breadth is much less than π, even after the furrow is evident. Active contraction of the surface layer, as demonstrated by Dan, Yamagita and Sugiyama (13), may provide a more adequate explanation of cleavage.

The movement of daughter cells by unretracted cell processes may be important in the arrangement of cells in histogenesis. The direction of the mitotic axis may also affect the orientation of cells in tissues. In the present work it has been found that the spindles of fibroblasts are eventually oriented in the direction of the radial spread of the outgrowth, as observed by Caffier (8). While the cause of this arrangement may be mechanical, for the birefringence of the solid medium indicates tension in the directions in which the culture is growing, it is not easy to understand how external forces would act to turn a rounded cell or the mitotic figure within it.

SUMMARY

1. Living fibroblasts in cultures of newt tissues have been examined by phase-contrast microscopy, and photographed serially during anaphase.

2. The separation of groups of daughter chromosomes takes place in two stages:

(a) the first movement is an approach by the chromosomes to the spindle poles:

(b) the second movement is due to elongation of the interchromosomal part of the spindle.

3. Cytoplasmic cleavage and the second anaphase movement begin simultaneously.

ADDENDUM

Since this paper was prepared for press, Jacquez and Biesele have published studies on chromosome separation and changes in cell shape during meiosis in the grasshopper *Psophus stridilus* (*Exptl. Cell Research*, **6**, 17 (1954)). Their graphs indicate that elongation of the spindle and decrease in cell width begin at the same time.

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OSMIUM TETROXIDE AND RUTHENIUM TETROXIDE AND THEIR REACTIONS WITH BIOLOGICALLY IMPORTANT SUBSTANCES

ELECTRON STAINS III

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Fixation with osmium tetroxide for electron microscopy (66, 54), and in particular for the electron microscopy of ultrathin sections, is at present unsurpassed in its ability to preserve tissue structures. The work of Fernandez-Morán (21), Palade (50), Porter (53), Rhodin (58), Sjöstrand (60) and many others emphasizes its unique role for electron microscopical work on biological material. Some authors have also made intensive studies of the conditions required to get the best results when osmium tetroxide is used as a fixative. Thus Palade recommended its application together with a buffer (50). Sjöstrand found a balanced solution of the fixative to give the best results (60). Rhodin studied extensively the effect of temperature and time on the fixation of kidney tissue (58). However, objections to the superiority of this fixative have been put forward (7).

Not only the excellent fixations with osmium tetroxide but also its contribution to the scattering power of the object, i.e. its contrast, are highly appreciated. In very thin sections with embedding material left in place, which is nowadays the rule, the osmium atom must be regarded as the only constituent giving significant contrast. A study of the contrast problems in ultrathin sections of biological material will be published elsewhere (6). In such sections of about 100–300 Å the differences in scattering power between the material to be studied and the embedding material can be neglected. Calculating with simplified equations the scattering power of an element is roughly proportional to Z^2, the square of the atomic number. This number is 76 for osmium. An average value for tissue and embedding material may be supposed to be 18. Thus osmium depositions in the tissue will play the decisive role in contrast formation, and, consequently, *in very thin sections only the distribution of the osmium can be seen.* It is therefore of particular interest to know what structures may be represented by the osmium deposits and this again will consequently be a question of the reaction of the osmium tetroxide with biological substances. On this account the present study is concerned with such reactions including the reactions of substances that may serve to elucidate the principal reactivity of OsO_4 towards biological substances.

Already in 1804 Tennant (62) and in 1814 Vauquelin (63) observed that a solution of "osmic acid" blackens organic substances by reduction to what they assumed to be metallic osmium. Later, in 1849, Branell (10) is reported to have made the first general study of the action of osmic acid, *De acidi osmici in homines et animalia effectu, commentatio physiologica,* but nothing exact is known about the results of this study. It was therefore Max Schultze (59) who was the first to introduce the tetroxide in histology and histochemistry, using the tetroxide during a study of the phosphorescent organ of the glow worm, *Lampyris splendidula,* on the assumption that the phosphorescence is dependent on oxygen uptake. In living phosphorescent animals the phosphorescent cells are strongly impregnated. Dead animals showed no osmium uptake in these cells. Later the tetroxide became a constituent of many histological fixing fluids (for historical data see (39)) but its use was restricted because it often interferes with staining techniques.

METHODS

0.01 molar osmium tetroxide solutions in either water or carbontetrachloride were used throughout this study. (0.01 ruthenium tetroxide solution was also tested at 20° C.) The substances tested were dissolved in distilled water or carbontetrachloride (analytical quality), whenever possible to a 0.1 molar solution. The reactions were performed at 0° C, 20° C and 50° C, using precooled or prewarmed test tubes, pipettes and solutions. 0.5 ml osmium tetroxide solution was mixed with 1 ml of the substance-solution to be tested and the reaction followed during certain time intervals. Substances available only in their hydrochlorides were tested both in this form and in alkaline solution. The alkalisation was achieved by adding the calculated amount of n/10 NaOH.

The readings are based on the fact that every change in colour, or the production of a black collodial solution or a precipitate, means a reaction with the osmium tetroxide. It is assumed that there is no reaction at all when the colourless (or very faint yellow) osmium tetroxide solution and the solution of the test substance do not show any visible alteration. This assumption does not hold true for proteins. Here often no obvious changes in colour or phase are observed, although the protein solutions are subject to slow alterations in their viscosity, their coagulability, etc.

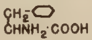
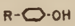
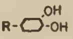
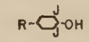
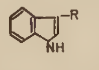
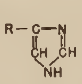
No special efforts have been made during this investigation to analyse the reaction products between osmium tetroxide and the organic compounds in question, some

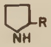
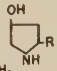
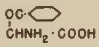
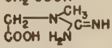
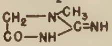
SUBSTANCE	PURI-TY	SOL.	FORMULA	0° C 0 1 2 16 24 48	20° C 0 1 2 3 4 5 7 16 20 24 48	50° C 0 1 2 16 24 48	COLOUR 0°	 20°	 50°	Ru
1 Methane	pract.	H_2O	CH_4	○○	○○	○◌			gy	⊛
Propane	"	"	C_3H_8	○○	○○	⊕⊕				⊛
Paraffine, liquid	pur.	CTCl		○○○○○○	○○○○○○○○○○○	○○○○○○				⊛
Paraffine, wax	pract.	"		○○○○○○	○○○○○○○○○○○	○◌◌◌◌◌			br	⊛
Ethylene	pract.	H_2O	C_2H_4 =	⊕⊛	⊕⊛	⊛⊛				⊛
Acetylene	"	"	C_2H_2 ≡	⊕⊛	⊕⊛	⊛⊛				⊛
2 Chloroform	puriss.	CTCl	CCl_3	○○○○○○	○○○○○○○○○○○	○○○○○○				
Carbontetrachloride	"	"	CCl_4	○○○○○○	○○○○○○○○○○○	○○○○○○				
Jodoform	"	H_2O	CJ_3	○○○○○○	○○○○○○○○○○○	○○○○○○			bl	⊛
3 Methanol	"	"	CH_3OH	○○○○○○	○○○○○○○○○◌◌	⊕⊕⊛⊛⊛⊛		gy		⊛
Ethanol	"	"	C_2H_5OH	○○○○⊖⊖	○⊖◌▨◍◍◍⊛⊛⊛⊛	○◍◍⊛⊛⊛	gy	bl	bk	⊛
Propyl- alcohol	"	"	C_3H_7OH	○◍◍◍◍◍	◌◍◍◍◍◍◍◍◍◍◍	○◍◍⊛⊛⊛	br	br ↓bk	br ↓bk	⊛
Butyl-n "	"	CTCl	C_4H_9OH	○▨▨▨◍⊛	○◍◍◍◍◍◍◍⊛⊛⊛	○◍⊛⊛⊛⊛	or ↓gy	bk	bk	⊛
Butyl-iso "	"	"	C_4H_9OH	○○⊖▨◍◍	○◍◍◍◍◍◍◍⊛⊛⊛	○◍◍⊛⊛⊛	gy	bk	bk	⊛
Amyl-iso "	"	"	$C_5H_{11}OH$	○○⊖▨◍◍	○○◌◌○▨▨◍◍◍◍	○◍◍◍◍◍	vt	vt ↓bk	br ↓bk	⊛
Hexyl alcohol, n	puriss.	CTCl	$C_6H_{13}OH$	○○○○⊖⊖	○○○○⊖⊖⊖⊖◌◌◌	○○⊖⊖▨◍	pk	pk	pk ↓bk	⊛
Heptyl " , n	pract.	"	$C_7H_{15}OH$	⊖⊖⊖⊖○○	⊖◌▨▨▨▨▨◍◍◍◍	○◌▨◍◍◍	gr	gr ↓bk	gr ↓bk	⊛
Octyl " , n	pur.	"	$C_8H_{17}OH$	○⊖⊖⊖○○	○⊖⊖⊖⊖⊖◌◌○◌○	○◌▨◍◍◍	pk ↓vt	pk ↓bk	vt ↓bk	⊛
Cetyl "	puriss.	"	$C_{16}H_{33}OH$	○○○⊖⊖○	○○○○○○○◌◌◌◌	○○○◌◌⊕	pr	gy	gy	⊛
Allyl "	"	H_2O	$CH_2OH \cdot CH : CH_2$ =	⊖⊛⊛⊛⊛⊛	▨◍⊛⊛⊛⊛⊛⊛⊛⊛⊛	▨⊛⊛⊛⊛⊛				⊛
Geraniol	pract.	CTCl	≐ =	⊖○○◍◍◍	◌◌◌○○○◌◌◌○○	○○○▨▨▨	br ↓bk	br	br	⊛
Nerol	"	"	= =	⊖○○○○○	◌◌◌◌◌◌◌◌◌◌◌	○○○○○○	br	br	br	⊛
Citronellol	"	"	=	⊖⊖○○○○	⊖⊖⊖⊖⊖⊖◌◌○○○	⊖⊖⊖○○○	pk ↓rd	pk ↓rd	pk ↓br	⊛
Glycerol	puriss.	H_2O	$C_3H_5(OH)_3$	○○○○○⊖	○○○○○○○⊖⊖◌◌	○⊖⊖⊖○⊕	ye	br	br	⊛
Ether	"	CTCl	$(CH_3)_2\cdot O$	○○○○○○	○○○○○○○○○○○	○○○⊖⊖⊖			ye	⊛
Dipropylether	"	"	$(C_3H_7)_2\cdot O$	○○○○○○	◌○○○○○◌◌○○○	○○○○○○	ye	ye	ye	⊛
Ethylenglucol	"	H_2O	$CH_2OH \cdot CH_2OH$	○○○○○○	○○○○○○○○○○⊖	○○○⊖⊖⊖		pk	pk	⊛
Dioxan	"	CTCl	$O<^{CH_2-CH_2}_{CH_2-CH_2}>O$	○○○○○○	○○○○○○○○○○○	○○○⊖○○			ye	⊛
4 Ammonium-sulfide	"	H_2O	$(NH_4)_2\cdot S$	⊛⊛⊛⊛⊛⊛	⊛⊛⊛⊛⊛⊛⊛⊛⊛⊛⊛	⊛⊛⊛⊛⊛⊛				⊛
Cysteine	"	alkal.	$CHNH_2\cdot CH\cdot SH$ $COOH$	○○⊛⊛⊛⊛	○○⊛⊛⊛⊛⊛⊛⊛⊛⊛	○⊛⊛⊛⊛⊛	br	br	br	⊛

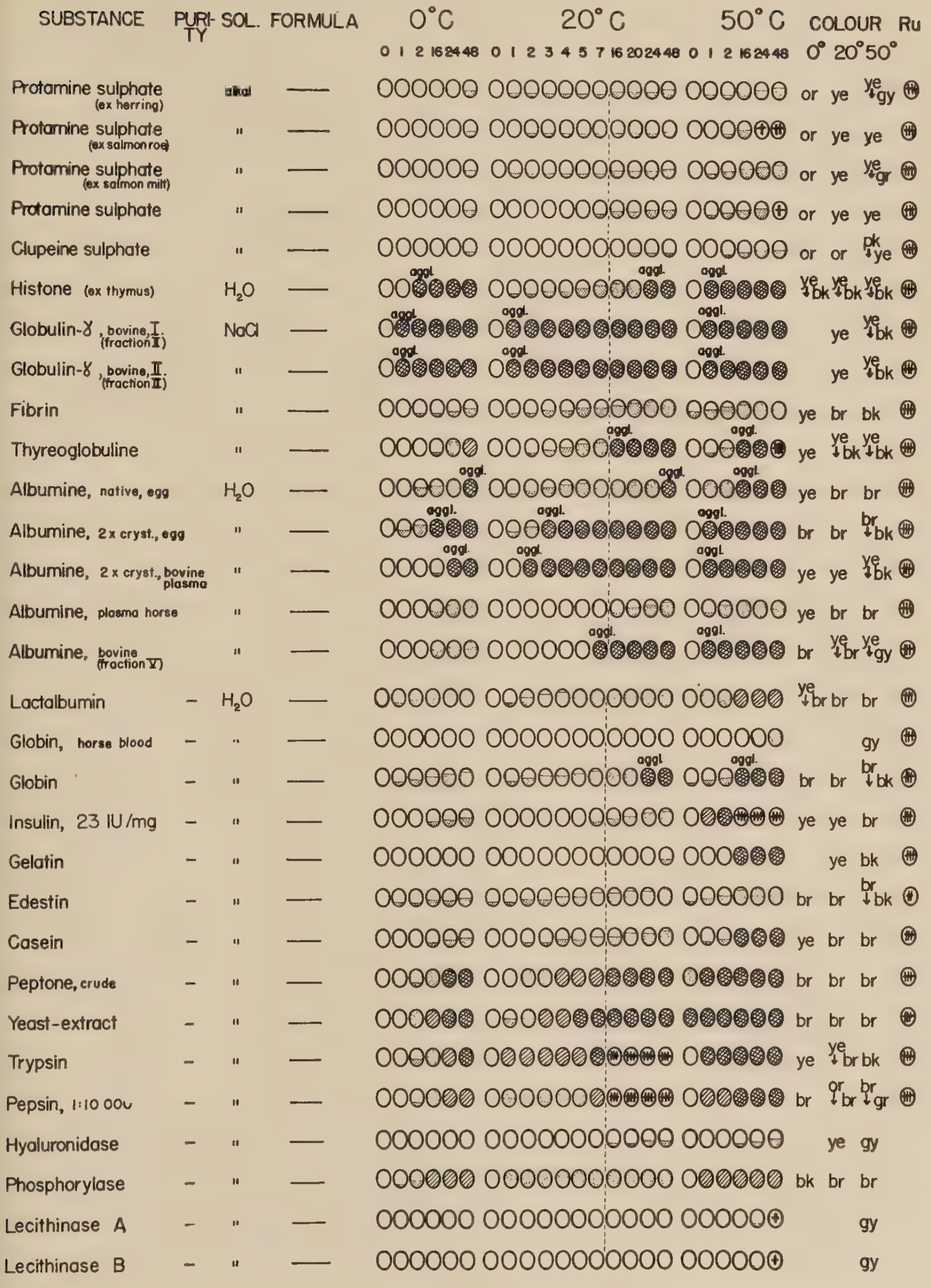
SUBSTANCE	PURITY	SOL.	FORMULA	0° C 0	1	2	16	24	48	20° C 0	1	2	3	4	5	7	16	20	24	48	50° C 0	1	2	16	24	48	COLOUR 0°	20°	50°	Ru
Cysteine-Cl	purss.	H_2O	CH_2SH $CHNH_2 \cdot COOH$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊘	br	br	br	⊛
Thioglucolic acid	pract.	"	$HOOC \cdot CH_2SH$	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛				⊛
Gluthathione	purss.	"	PEPT.–SH	○	⊛	⊛	⊛	⊛	⊛	○	●	●	●	●	●	●	●	●	●	●	○	●	●	●	●	●		br ↓bk	br ↓bk	⊛
Cystine	"	"	R–S–S–R	○	○	○	○	◍	⊘	○	○	○	○	○	○	○	⊘	⊘	⊘	⊘	○	⊘	⊘	⊘	⊘	⊘	br	br	br	⊛
5 Hydrazine	pur.	alkal.		●	⊛	⊛	⊛	⊛	⊛	⊘	⊘	⊘	⊘	⊘	⊘	⊘	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	bk	br		⊛
Hydrazine-Cl	"	H_2O	$H_2N \cdot NH_2$	◍	●	●	●	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	br			⊛
Methylamine	purss.	alkal.		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊖	○	○	⊖	⊕	⊛	⊛		ye	pk	⊛
Methylamine-Cl	"	H_2O	$CH_3 \cdot NH_2$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊖	⊖			gy	⊛
Ethylamine	"	"	$C_2H_5 \cdot NH_2$	◍	◍	◍	○	◍	◍																		br			⊛
Heptylamine	"	CTCl	$C_7H_{15} \cdot NH_2$	⊛	⊛	⊛	⊛	⊛	⊛	◍	◍	◍	◍	◍	◍	◍	◍	◍	◍	◍	◍	◍	◍	◍	○	◍		br	br	⊛
Diethylamine	pur.	"	$(C_2H_5)_2 \cdot NH$	○	⊘	●	⊛	⊛	⊛	⊘	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	br	br		⊛
Hydroxylamine	purss.	alkal.		○	⊖	◍	◍	◍	◍	○	⊖	◍	◍	◍	◍	◍	◍	◍	○	○	⊖	○	○	○	○	○	or	or	or	⊕
Hydroxylamine-Cl	"	H_2O	$NH_2 \cdot OH$	○	○	○	⊖	⊖	⊖	○	⊖	⊖	⊖	⊖	○	○	○	○	○	○	⊖	○	○	○	○	○	or	or	or	⊕
6 Formaldehyde	pur.	"	HCHO	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊛	⊛	⊛				⊛
Oenanthal	purss.	CTCl	$CH_3(CH_2)_5 \cdot CHO$	○	○	○	○	○	○	○	○	○	◍	○	○	○	○	○	○	○	○	○	○	◍	○	◍	br	br	br	⊛
Trioxymethylene	purss.	H_2O	$(HCHO)_3$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊕	⊛				⊛
Acetone	"	CTCl	$CH_3CO \cdot CH_3$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊕	⊕	⊛				⊛
Diacetyl	"	"	$CH_3 \cdot CO \cdot CO \cdot CH_3$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊖	⊖	⊖	○	⊛	⊛	⊛	⊛	⊛		ye		⊛
Acetylacetone	"	"	$CO \cdot CH_3$ $CH_2\ CO \cdot CH_3$	○	○	⊖	⊘	⊘	⊘	○	○	○	⊘	⊘	⊘	⊘	●	●	●	●	○	●	●	●	●	●	br	br	br	⊛
7 Urea	"	H_2O	$OC \cdot (NH_2)_2$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○				⊛
Guanidine	pur.	alkal.		○	○	○	○	○	⊖	⊖	⊖	⊖	⊖	⊖	⊖	⊖	⊖	⊖	⊖	⊖	⊖	⊖	⊖	⊖	⊖	⊖	pk	ye ↓or	pk	⊛
Guanidine-Cl	"	H_2O	$NHC \cdot (NH_2)_2$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊖	○	○	○	⊖	⊖	⊖		pk	pk	⊛
Thiourea	purss.	"	$SC\ (NH_2)_2$	○	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛				⊛
8 Ethanolamine	"	CTCl	$CH_2NH_2 \cdot CH_2OH$	○	⊖	⊖	⊖	⊖	◍	⊖	⊖	◍	◍	◍	◍	◍	◍	◍	◍	◍	⊖	◍	◍	◍	◍	◍	ye	gr ↓br	gr	⊛
Choline	"	alkal.	CH_2OH $CH_2N(CH_3)_3\ OH$	○	○	○	○	○	●	⊖	⊖	◍	◍	⊘	⊘	●	●	●	●	●	⊖	●	●	⊛	⊛	⊛	vt	pk ↓bk	pk ↓bk	⊛
Choline-Cl	"	H_2O		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○				⊛
Triethanolamine	pur.	CTCl	$(C_2H_4OH)_3 \cdot N$	○	◍	◍	◍	○	○	◍	◍	◍	◍	◍	◍	◍	◍	◍	◍	◍	◍	◍	◍	◍	◍	◍	br	br	br	⊛
9 Methylformate	purss.	"	$HCOOCH_3$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊖	○	○	○	⊘	●	⊛		gy	bk	⊛
Ethylacetate	"	"	$C_2H_5OOCCH_3$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊕	⊕	⊕				⊛
10 Coconut fat	-	"	—	○	◍	◍	●	●	●	○	◍	◍	◍	⊘	⊘	●	●	●	●	●	○	◍	◍	●	●	●	br ↓bk	br ↓bk	br ↓bk	⊛

	SUBSTANCE	PURITY	SOL.	FORMULA	0° C	20° C	50° C	COLOUR 0°	20°	50°	Ru
					0 1 2 16 24 48	0 1 2 3 4 5 7 16 20 24 48	0 1 2 16 24 48				
	Lard	–	CTCl	—	○○○◍◍◍	○○○⊘⊘⊘⊘◍◍◍◍	○◍◍◍⊛⊛	bk	bk	bk	⊛
	Butter	–	"	—	○◌◌◍◍◍	◌⊘⊘⊘⊘◍◍⊛⊛⊛⊛	○⊛⊛⊛⊛⊛	bk	bk	bk	⊛
	Peanut- oil	–	"	—	◌○○○○○	○○○○○○○◌○○○	○○○○○○	br	br	br	⊛
	Olive – "	–	"	—	◌◌◌◌◌◌	◌○○○○○○○○○○	◌◌○○○○	br	br	br	⊛
	Castor – "	–	"	—	◌◌◌◌◌◌	○○○○○○○○○○○	○○○○○◌	br	br	br	⊛
	Lavender-"	–	"	—	○○○○○○	○○○○○○○◌○○○	◌○○○○○	br	br	br	⊛
	Clove – "	–	"	—	○○⊘⊘⊘◍	○○○○○○○◌○○○	○○○○⊘⊘	br	br	br	⊛
11	Formic acid	purss.	H_2O	$HCOOH$	○○○○○○	○○○○○○○○○○○	○○○○○○			pk	⊖
	Acetic "	"	"	$CH_3\ COOH$	○○○○○○	○○○○○○○○○○○	○○○○○○				⊖
	Propionic "	"	"	$C_2H_5\cdot COOH$	○○○○○○	○○○○○○○○○○○	○○○○○○				⊖
	Butyric "	pur.	"	$C_3H_7\cdot COOH$	○○○○○○	○○○○○○○○○○○	○○○⊘⊘⊘	gy	gy	bk	⊛
	Valeric "	"	"	$C_4H_9\cdot COOH$	○○○○⊖○	○○○○○○○○○○○	○○⊖○⊘⊘	pk	gy	gy	⊛
	Valeric "	"	CTCl		○○○○○⊖	○○○○○○○⊖⊖⊖○	○○⊖⊖○○	vt	vt	vt	⊛
	Caproic "	"	"	$C_5H_{11}\cdot COOH$	○○○○○⊘	○○○⊖⊖⊖⊖○⊕⊛⊛	○⊖⊖⊘◍⊛	vt	vt	vt	⊛
	Caprylic "	purss.	"	$C_7H_{15}\cdot COOH$	○○○○○⊘	○○⊖⊖⊖⊖⊖○○⊘⊘	○○○⊘⊘⊘	vt	vt	vt	⊛
	Palmitic acid	pur.	CTCl	$C_{15}H_{31}\cdot COOH$	○○○○○○	○○○○○○○○○○○	○○○○○○				⊛
	Stearic "	"	"	$C_{17}H_{35}\cdot COOH$	○○○○○○	○○○○○○○○○○○	○○○○○○				
	Oleic "	"	"	$C_{17}H_{33}\cdot COOH =$	◌◌◌◌◌◌	◌◌◌◌◌◌◌◌◌◌◌	○○○○○○	br	br	br	⊛
	Linoleic "	"	"	$C_{17}H_{31}\cdot COOH = =$	○○○⊘⊘⊘	◌◌◌◌◌◌◌◍◍◍◍	⊘⊘⊘◍◍◍	br	br	br	⊛
	Linolenic "	"	"	$C_{17}H_{29}\cdot COOH \overset{=}{= =}$	○○○◌◌◌	◌◌◌◌◌◌○◍◍◍◍	⊘⊘⊘◍◍◍	br	br	br	⊛
	Arachidonic "	"	"	$C_{19}H_{31}\cdot COOH \equiv\equiv$	○○○○◌◌	◌◌⊘⊘⊘⊘⊘⊘⊘⊘◍	⊘⊘⊘⊘⊘⊘	br	br	br	⊛
12	Lactic "	purss.	H_2O	$CH_3\cdot CHOH\cdot COOH$	○○○○⊘⊘	○⊖⊖◌◌◌◌◌◌◌◌	○⊘◌◌○○	br	br ↓gr	br ↓gr	⊛
	Pyruvic "	"	"	$CH_3\cdot CO\cdot COOH$	○○⊖◌◌◌	⊖⊛⊛⊛⊛⊛⊛⊛⊛⊛⊛	⊛⊛⊛⊛⊛⊛	pk ↓or ye			⊛
13	Oxalic "	"	"	$COOH\cdot COOH$	○○○○○○	○○○○○○○○○○○	○○○○○○				⊖
	Malonic "	"	"	$COOH$ $CH_2\ COOH$	○○○○○○	○○○○○○○○○○○	○○○⊕⊕⊛				⊛
	Succinic "	"	"	$COOH$ $(CH_2)_2\ COOH$	○○○○○○	○○○○○○○○○○○	○○○○○○				⊖
	Glutaric "	"	"	$COOH$ $(CH_2)_3\ COOH$	○○○○○○	○○○○○○○○○○○	○○○⊖◌⊛			bl	⊕
	Adipic "	"	"	$COOH$ $(CH_2)_4\ COOH$	○○○○○○	○○○○○○○○○○○	○○○○○○				⊖
	Pimelic "	pur.	"	$COOH$ $(CH_2)_5\ COOH$	○○○○○○	○○○○○○○○○○○	○○○○⊕⊛				
	Suberic "	"	"	$COOH$ $(CH_2)_6\ COOH$	○○○○○○	○○○○○○○○○○○	○○○○○○				⊖

SUBSTANCE	PURITY	SOL.	FORMULA	0°C						20°C											50°C						COLOUR			Ru
				0	1	2	16	24	48	0	1	2	3	4	5	7	16	20	24	48	0	1	2	16	24	48	0°	20°	50°	
Malic acid	purss.	H_2O	$CH_2\cdot COOH$ $CHOH\cdot COOH$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	◍	◍	◍			br	⊛
Tartaric "	"	"	COOH $(CHOH)_2\cdot COOH$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	◒	◍			gr	⊛
Citric "	"	"	$CH_2\cdot COOH$ $COH\cdot COOH$ $CH_2\cdot COOH$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	◍	○	◍			br ↓gr	⊖
14 Glyceraldehyde, dl	"	"	CHO $CH_2OH\cdot CH_2OH$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊛	⊛				⊛
Xylose, d	"	"	CHO $(CHOH)_3\cdot CH_2OH$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	◍	⊘	⊘			bk	⊛
Arabinose, l+	"	"	"	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○				⊕
Ribose, d-	"	"	"	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	◒	○	○	○	◍	◍	◍		ye	ye ↓bk	⊛
Glucose	"	"	CHO $(CHOH)_4\cdot CH_2OH$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○				⊕
Mannose, d+	"	"	"	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○				⊕
Galactose, d+	"	"	"	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○				⊕
Fructose, d-	"	"	$CO\cdot CH_2OH$ $(CHOH)_3\cdot CH_2OH$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	◒	⊘			gy	⊛
Galacturonic acid, d+	"	"	CO COOH $(CHOH)_3\cdot CH_2OH$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	◍			gy	⊛
Mucic "	"	"	COOH $(CHOH)_4\cdot COOH$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊘	●	●			bk	⊛
Glucosamine	"	alkal.	$CHNH_2\cdot CHO$ $(CHOH)_3\cdot CH_2OH$	○	○	○	○	○	◒	○	○	○	○	◒	◒	◒	◒	◒	◍	◍	◒	◒	◒	◍	◍	⊘	ye	ye	ye ↓br	⊛
Glucosamine-Cl	"	H_2O		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	◍			ye	⊛
Heparin	purss.	H_2O	—	○	○	○	○	○	◒	○	○	○	○	○	○	○	○	◒	◒	◒	○	○	○	◒	◍	◍	ye	ye	gy ↓ye	⊛
Fructose diphosphate, 1,6	"	"	$CHO\cdot PO_3H_2$ $(CHOH)_4$ $CHO\cdot PO_3H_2$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊕	⊛				⊛
Na- -glycerophosphate	"	"	CH_2OH CH_2OH $CHO\cdot PO_3H_2$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊘	⊘	⊘			gy	⊛
Succrose	"	"	$C_{12}H_{22}O_{11}\cdot H_2O$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○				⊕
Maltose	"	"	"	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○				⊕
Cellobiose	"	"	"	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊘	⊘	⊘			gy	⊕
Lactose	"	"	"	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊘			gy	⊕
Gum arabic	pur.	"	—	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	◒	◒			ye	⊛
Dextrin	"	"	$(C_6H_{10}O_5)_x$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○				⊕
Starch	"	"	"	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	◒	⊘	⊘	⊘			gy	⊛
Glycogen	"	"	"	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊘			gy	⊛
Inulin	"	"	"	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊘			gy	⊛
Pektin	"	"	—	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊘	○	○	○	⊘	⊘	⊘			gy	⊛
Cellulose	"	"	$(C_6H_{10}O_5)_x$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	◒	◒	◒	⊘			gy	⊛
Lignin	—	methanol	—	○	○	○	○	○	○	○	○	○	○	○	○	○	⊘	⊘	⊘	⊘	○	○	◍	⊛	⊛	⊛		gy	gy	⊛

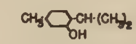
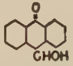
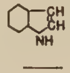
SUBSTANCE	PURITY	SOL.	FORMULA	0°C						20°C											50°C						COLOUR			Ru
				0	1	2	16	24	48	0	1	2	3	4	5	7	16	20	24	48	0	1	2	16	24	48	0°	20°	50°	
15 Glycine	purss.	H_2O	$CH_2NH_2 \cdot COOH$	○	○	○	○	⊖	○	○	○	○	○	○	○	○	○	○	⊖	⊖	○	○	○	○	●	●	ye	ye	vt	⊞
Alanine, α	"	"	$CH_3CHNH_2 \cdot COOH$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊖	◌	○	○	○	●	●	●		vt	vt	⊞
Alanine, β	pur.	"	$CH_2NH_2 \cdot CH_2 \cdot COOH$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●			bk	⊞
Serine	pract.	"	$CH_2 \cdot COOH$ $CHNH_2 \cdot COOH$	○	○	○	○	○	○	○	○	○	○	○	○	○	●	●	●	●	○	●	●	●	●	●		bl	vt	⊞
Phenylalanine	pur.	"		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	◌	◍	◍			bk	⊞
Tyrosine, dl-	purss.	"		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊖	⊖	○	⊖	◌	⊞	⊞	⊞		gy	gy	⊞
Dioxy-phenylalanine	"	"		○	●	●	●	●	●	◌	●	●	●	●	●	●	●	●	●	●	◌	●	●	●	●	●	bl	bl	bl	⊞
Diiodotyrosine	"	"		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊕	⊞	⊞				⊞
Tryptophane, l-	"	"		○	⊖	⊖	◍	◍	⊞	○	○	◌	⊞	⊞	⊞	⊞	⊞	⊞	⊞	⊞	○	◍	●	●	●	●	pk ↓br	pk ↓br	bk	⊞
Histidine, Cl	"	H_2O		○	○	⊖	○	⊕	⊕	○	○	○	○	○	○	○	●	●	●	●	◌	●	●	●	●	●	gy	vt	vt	⊞
Histidine	"	alkal.		○	○	⊖	●	●	⊞	○	◌	⊞	⊞	⊞	⊞	⊞	⊞	⊞	⊞	⊞	○	⊞	⊞	⊞	⊞	⊞	ye ↓rd	br		⊞
Histidine	"	alkal.		○	○	◌	◍	●	⊞	○	◌	●	●	●	●	●	⊞	⊞	⊞	⊞	○	⊞	⊞	⊞	⊞	⊞	ye ↓rd	br		⊞
Histidine, Cl_2	"	H_2O		○	○	○	⊖	⊖	⊖	○	○	○	○	○	○	○	●	●	●	●	◌	●	●	●	●	●	pk	vt	vt	⊞
Amino-butyric acid	pract.	"	CH_2CH_3 $CHNH_2 \cdot COOH$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	◍	●			bl	⊞
Methionine, dl-	purss.	"	$CH_2CH_2 \cdot SCH_3$ $CHNH_2 \cdot COOH$	○	◌	◍	●	●	●	◌	◍	◍	◍	◍	◍	●	●	●	●	●	◌	●	●	●	●	●	br	br	br	⊞
Valine, dl-	"	"	$CH-(CH_3)_2$ $CHNH_2 \cdot COOH$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	◍	◍	◍			gy	⊞
Norvaline, dl	purss.	H_2O	$(CH_2)_2 \cdot CH_3$ $CHNH_2 \cdot COOH$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○			pk	⊞
Leucine, l	"	"	$CH \cdot (CH_3)_2$ $CH_2 \cdot CHNH_2 \cdot COOH$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○				⊞
Iso-leucine, dl	"	"	$CH_3 \; C_2H_5$ $CH \cdot CHNH_2 \cdot COOH$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	◍	◍	⊞			vt	⊞
Norleucine, dl	"	"	$(CH_2)_3 \cdot CH_3$ $CHNH_2 \cdot COOH$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊖	⊖	○			ye	⊞
Arginine, l+	"	alkal.		○	⊖	◌	●	●	●	◌	●	●	●	●	●	●	●	●	●	●	○	●	●	●	●	●	br ↓vt	br ↓vt	br	⊞
Arginine-Cl, l+	"	H_2O	$NH_2 \cdot NH \cdot CNH-R$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	◍	◍	○	○	○	○	◍	◍		rd	rd	⊞
Ornithine	"	alkal.		○	○	○	●	●	●	○	●	●	●	●	●	●	●	●	●	●	○	●	●	●	●	●	bk	bk	bk	⊞
Ornithine-Cl_2	"	H_2O	$(CH_2)_2 \cdot CH_2NH_2$ $CHNH_2 \cdot COOH$	○	○	○	○	⊖	⊖	○	○	○	○	○	○	○	⊖	○	◍	●	○	⊖	●	●	●	●	pk	pk ↓bl	pk ↓bl	⊞
Citrulline, l+	"	"	$(CH_2)_3 \cdot NH \cdot CONH_2$ $CHNH_2 \cdot COOH$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	◍	○	○	○	○	◍	●		gy	bk	⊞
Lysine	"	alkal.		○	⊖	◌	●	●	●	○	●	●	●	●	●	●	●	●	●	●	○	●	●	●	●	●	br	br	br	⊞
Lysine-Cl	"	H_2O		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	○	○	○	◍	●	●		br	bk	⊞
Lysine	"	alkal.	$(CH_2)_3 \cdot CH_2NH_2$ $CHNH_2 \cdot COOH$	○	⊖	◌	◍	◍	◍	○	◍	◍	◍	◍	◍	◍	●	●	●	●	○	◍	◍	●	●	●	br	br	br	⊞
Lysine-Cl_2	"	H_2O		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	◍	●	⊞		gy	bk	⊞
Aspartic acid	"	"	$CH_2 \cdot COOH$ $CHNH_2 \cdot COOH$	○	○	○	○	○	○	○	○	○	○	○	○	○	○	◍	◍	●	○	○	○	●	●	●	pk	vt	vt	⊞

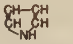
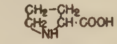
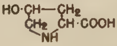
SUBSTANCE	PURITY	SOL.	FORMULA	0°C	20°C	50°C	COLOUR			Ru
				0 1 2 16 24 48	0 1 2 3 4 5 7 16 20 24 48	0 1 2 16 24 48	0°	20°	50°	
Glutamic acid, Cl	pract.	H_2O	$(CH_2)_2 \cdot COOH$ $CHNH_2 \cdot COOH$	○○○◍◍◍	○◍◍◍◍◍◍◍◍◍◍	○◍◍◍◍◍	br	br	br	⊛
Glutamic acid	"	alkal.		○○○○⊛⊛	○○○○○○○⊘⊘◍◍	○○○⊘◍◍		bl	bl	⊛
Threonine, dl	purss.	H_2O	$CHOH \cdot CH_3$ $CHNH_2 \cdot COOH$	○○○○○○	○○○○○○○○○◒⊘	○◒○○○○		bl	vt	◉
Proline, dl	"	"		○○○◍◍◍	○○○⊘⊘◍◍◍◍◍◍	○⊘◍◍◍◍	bk	bk	br	⊛
Oxyproline, l	"	"		○○○◍◍◍	○○⊘◍◍◍◍◍◍◍◍	○◍◍◍◍◍	bk	bk	bk	⊛
Asparagine, l	"	"	$CH \cdot CONH_2$ $CHNH_2 \cdot COOH$	○○◒◍◍◍	○◒◍◍◍◍◍◍◍◍◍	○◍◍◍◍◍	vt	vt	br ↓vt	⊛
Glutamine, l	"	"	$(CH_2)_2 \cdot CONH_2$ $CHNH_2 \cdot COOH$	○○○○○○	○○○○○○○○○◒◒	○○○◍◍◍		gy	vt	◉
Betaine, Cl	"	"	$CH_2 \cdot \underset{+}{\overset{-}{N}}(CH_3)_3$ $CO \cdot O$	○○○○○○	○○○○○○○○○○○	○○○○◒◒			gy	⊛
Betaine	"	alkal.		○○○○○○	○○○○○○○○○○○	○○○○○○				⊛
Hippuric acid	"	H_2O		○○○○○○	○○○○○○○○○○○	○○○○○○				◉
Cystine, l	"	"	R–S–S–R	○○⊘◍◍◍	○⊘◍◍◍◍◍◍◍◍◍	○◍◍◍◍◍	br	br	br	⊛
Cysteine, l Cl	"	"	$CH_2 \cdot SH$ $CHNH_2$ $COOH$	○○○○○○	○○○○○○○○○○○	○○○○○○	br	br	br	⊛
Cysteine, l	"	alkal.		○○⊛⊛⊛⊛	○○⊛⊛⊛⊛⊛⊛⊛⊛⊛	○⊛⊛⊛⊛⊛	br	br	br	◉
Sarcosine	"	H_2O	$NH \cdot CH_3$ $CH_2 \cdot COOH$	○○○◍◍◍	○○⊘⊘⊘⊘⊘⊘⊘⊘⊘	◒◍◍◍◍◍	gr ↓vt	br	vt	⊛
Creatine	"	"		○○○○○○	○○○○○○○○○○○	○○○○◒⊘			gr	⊛
Creatinine	"	"		○○○○○○	○○○○○○○◒○○○	○○○⊘◍⊛		or ↓br	or ↓br	⊛
16 Glycyl-glycine	purss.	H_2O	——	○○○○○○	○○○○○○○◍◍◍◍	○⊘⊘○○○	gr	gr ↓bk	bk ↓br	◉
Glycyl-leucine	"	"	——	○○○○○○	○○○○○○○○○○○	○○○○○○		pk	br	⊛
Alanyl-glycine	"	"	——	○○○◒◒○	○○○◒○○○◍◍◍◍	○○○○○○	ye ↓br	ye ↓gr	br	⊛
Glycyl-l-tyrosine	"	"	——	○○○○○○	○○○○○○○○○◒○	○○○○⊘⊘		ye	br	⊛
Glycyl-l-tryptophane	"	"	——	○◒◒◍◍◍	○◒⊘◍◍◍◍◍◍◍◍	○◍◍◍◍◍	pk ↓bk	br ↓bk	pk ↓bk	⊛
dl-Leucyl-valine	"	"	——	○○○○○○	○◒◒◒◒◒○◍◍◍◍	◒○◍◍◍◍	ye ↓br	ye ↓br	ye ↓br	⊛
Glutathione	"	"	——	○⊛⊛⊛⊛⊛	○◍◍◍◍◍◍◍◍◍◍	○◍◍◍◍◍	vt	bk	bk	⊛
Glutathione	"	H_2O_2 treated	—	○○○○○○	○○○○○○○○○○○	○○○○○○				⊛
Ergotamin	"	H_2O	——	○○○⊛⊛⊛	○⊘⊘⊘⊘⊘◍⊛⊛⊛⊛	○⊘⊘⊛⊛⊛	vt	br	bk	⊛
Penicillin	pur.	"	——	○○○○○⊘	○○○○○○○⊘⊘◍◍	○◒○◍◍◍	gy	bk	bk	⊛
17 Protamine sulphate (ex herring)	—	"	——	○○○○⊘⊘	○○○○○○○○○◒◒	○○○○◒◒	or	or	or ↓ye	⊛
Protamine sulphate (ex salmon roe)	—	"	——	○○○○⊘⊘	○○○○○○○○○○○	○○○○○○	ye	ye	ye ↓gr	⊛
Protamine sulphate (ex salmon milt)	—	"	——	○○○○⊘⊘	○○○○○○○○○○○	○○○○○○	ye	ye	ye	⊛
Protamine sulphate	—	"	——	○○○○⊘⊘	○○○○○○○○○○○	○○○○○○	ye	ye	ye	⊛
Clupeine sulphate	—	"	——	○○○○○○	○○○○○○○○○○○	○○○○⊘⊘	ye	ye	ye ↓gy	⊛


0°C
0 1 2 16 24 48
20° C
0 1 2 3 4 5 7 16 20 24 48
50° C
0 1 2 16 24 48
aggl.

SUBSTANCE	PURITY	SOL.	FORMULA	COLOUR 0°	COLOUR 20°	COLOUR 50°	Ru
Protamine sulphate (ex herring)		alkal	—	or	ye	ye ↓ gy	⊕
Protamine sulphate (ex salmon roe)		"	—	or	ye	ye	⊕
Protamine sulphate (ex salmon milt)		"	—	or	ye	ye ↓ gr	⊕
Protamine sulphate		"	—	or	ye	ye	⊕
Clupeine sulphate		"	—	or	or	pk ↓ ye	⊕
Histone (ex thymus)		H_2O	—	ye ↓ bk	ye ↓ bk	ye ↓ bk	⊕
Globulin-γ, bovine, I. (fraction I)		NaCl	—		ye	ye ↓ bk	⊕
Globulin-γ, bovine, II. (fraction II)		"	—		ye	ye ↓ bk	⊕
Fibrin		"	—	ye	br	bk	⊕
Thyreoglobuline		"	—	ye	ye ↓ bk	ye ↓ bk	⊕
Albumine, native, egg		H_2O	—	ye	br	br	⊕
Albumine, 2 x cryst., egg		"	—	br	br	br ↓ bk	⊕
Albumine, 2 x cryst., bovine plasma		"	—	ye	ye	ye ↓ bk	⊕
Albumine, plasma horse		"	—	ye	br	br	⊕
Albumine, bovine (fraction V)		"	—	br	ye ↓ br	ye ↓ gy	⊕
Lactalbumin	–	H_2O	—	ye ↓ br	br	br	⊕
Globin, horse blood	–	"	—			gy	⊕
Globin	–	"	—	br	br	br ↓ bk	⊕
Insulin, 23 IU/mg	–	"	—	ye	ye	br	⊕
Gelatin	–	"	—		ye	bk	⊕
Edestin	–	"	—	br	br	br ↓ bk	⊕
Casein	–	"	—	ye	br	br	⊕
Peptone, crude	–	"	—	br	br	br	⊕
Yeast-extract	–	"	—	br	br	br	⊕
18 Trypsin	–	"	—	ye	ye ↓ br	bk	⊕
Pepsin, 1:10 000	–	"	—	br	or ↓ br	br ↓ gr	⊕
Hyaluronidase	–	"	—		ye	gy	
Phosphorylase	–	"	—	bk	br	br	
Lecithinase A	–	"	—			gy	
Lecithinase B	–	"	—			gy	

	SUBSTANCE	PURITY	SOL.	FORMULA	0°C 0 1 2 16 24 48	20°C 0 1 2 3 4 5 7 16 20 24 48	50°C 0 1 2 16 24 48	COLOUR 0°	COLOUR 20°	COLOUR 50°	Ru
	RN-ase	purss.	H_2O	—	○○○○◒◒	○○○○○◒◒◒◒◒●	○○◒●●●	br	br	br	⊛
	DN-ase	"	"	—	○○○○○○	○○○○○◒●●●●● aggl.	○○◒●●● aggl.		ye	ye↓br	⊛
19	RNA I	—	"	—	○○○○○○	○○○○○○○○○○○	○○○◒◒◒			gy	⊛
	RNA II	—	"	—	○○○○○○	○○○○○○○○○○○	○○○○○○				⊛
	Sodium-nucleinate	—	"	—	○○○○○○	○○○○○○○○○○○	○○○○○○			gy	⊛
	DNA I	—	"	—	○◒◒◍◍◍	○◒◒◒◒◒◒◍◍◒◒	○◒◒◍○⊘	ye	ye↓gy	vt↓bk	⊛
	DNA II	—	"	—	○○○○○○	○○○○○○○○○○○	○○◒◍◒◒			ye↓bl	⊛
20	Pyridine	pur.	CTCl	—	⊘⊘⊘⊕⊕⊕	⊘⊘⊘⊘⊘⊘⊘⊛⊛⊛⊛	⊘⊘⊘⊛⊛●	ye	ye	ye	⊛
	Nicotinamide	purss.	H_2O	—	○○○○○○	○○○○○○○○○○○	○○○○○○				⊛
	Uracil	"	"	—	○○○○○○	○○○○○○○○○○○	○○○◍◍◍			gy	⊛
	Cytosine	"	"	—	○○○○○○	○○○○○○○○○○◒	○○○◍◍◍		pk	gy	⊛
	Thymine	"	"	—	○○○○○○	○○○○○○○○◒◍◍	○○◍◍◍◍		gy↓bl	gr↓bl	⊛
	Uric acid I	"	"	—	○○○◒◒◒	○○○◒◍◍◍◍◍◍⊘	○◍○⊘⊛⊕	ye	gy	bl↓bk	⊛
	Uric acid II	"	Sod. acet.	—	○○○⊛⊛●	○◍◍◍◍◍◍⊛⊛⊛⊛	○⊘⊘⊛⊛⊛		or↓br	or↓bk	⊛
	Alloxan	"	H_2O	—	○○○○○○	○○○○○○○○○○○	○○○○○○				⊕
	Allantoin	purss.	H_2O	—	○○○○○○	○○○○○○○○○○○	○○○○○○				⊕
	Xanthine	"	"	—	○○○○○○	○○○○○○○○○○○	○○○○○◒			gy	⊕
	Hypoxanthine	"	"	—	○○○○○○	○○○○○○○○○○○	○○○○○◍			gy	⊕
	Adenine	"	"	—	○○○○○○	○○○○○○○○○○○	○◒◍⊛⊛⊛			pk	⊛
	Guanine	"	"	—	○○○○○○	○○○○○○○○○○○	○○○○○○				⊕
	Guanosine	"	"	—	○○○○○○	○○○○○○○○○○○	○○○○○○				⊕
	Adenosine	"	"	—	○○○○○○	○○○○○○○○○○○	○○○○○○				⊕
	Adenosine-3-phosphoric acid	pur.	"	—	○○○○○○	○○○○○○○○○○○	○○○○⊕⊕				
	Adenosine-5-phosphoric acid	purss.	"	—	○○○○○○	○○○○○○○○○○○	○○○○○○				
	Adenosine-triphosphate	"	"	—	○○○○○○	○○○○○○○○○○○	○○○○◒◒			gy↓vt	
21	Benzene	"	CTCl	C_6H_6	◒◒◒◒◒◒	◒◒◒◒◒◒◒◒◒◒◒	◒◒◒◒◒◒	gr	gr	gr	⊛
	Naphtalene	"	"	$C_{10}H_8$	○○◒◒◒◒	◒◒◒◒◒◒◒◒◒◒◒	◒◒◒◒◒◒	ye	ye	ye	⊛
	Anthracene	pract.	"	$C_{14}H_{10}$	○⊛⊛⊛⊛⊛	○○◒◒◒◒◍◍◍◍◍	○○◍◍◍◍		gy	bk	⊛
	Chlorobenzene	pur.	"	C_6H_5Cl	○○○○○○	○○○○○○○○○○◒	◒◒◍◍○◍		gy	gy	⊕
	Bromobenzene	"	"	C_6H_5Br	○◒◒◍◍◍	○◒◒◒◍◍◍⊕⊕⊕⊛	○◒◒⊛⊛⊛	gy	ye	gr	⊛

SUBSTANCE	PURITY	SOL.	FORMULA	0° C	20° C	50° C	COLOUR 0°	COLOUR 20°	COLOUR 50°	Ru
				0 1 2 16 24 48	0 1 2 3 4 5 7 16 20 24 48	0 1 2 16 24 48	0°	20°	50°	
Phenol		H_2O	$C_6H_5 \cdot OH$	○○○○○○	○○○○○○○○○○○	○○○○○○			gy	⊕
Naphtol-α		"	$C_{10}H_7 \cdot OH$	○○○◌◌◌	○○○○○○○◌◌◌●	○○○○◍◍	vt	bl	bl	⊕
Naphtol-β		"		○○○◒◌◌	○○○○○○○◒◒◌◌	○○○◌◌◌	gy	pk ↓gr	gr ↓bl	⊕
Thymol		"		○◒◒◒◒◒	◒◒◒◒◒◒◒◒◌◌◌	◒◒◒◌○◌	pk	pk ↓or	pk ↓or	●
Resorcinol		"	$C_6H_4(OH)_2$	○○○○◒◍	○○○◒◒◒◒◉◉◉◉	○◒◍◉◉◉	bk	bk	bk	●
Hydroquinone		"	"	●●●●●●	●●●●●●●●●●●	●●●●●●				●
Pyrogallol		"	$C_6H_3(OH)_3$	○◍◍◉◉◉	○◍◍◍◍◉◉◉◉◉◉	○◍◉◉◉◉	bl	bl	bl	●
Phloroglucinol		"	"	○◒◌◍◉◉	○◉◉◉◉◉◉◉◉◉◉	◒◉◉◉◉◉	bk	bk	bk	●
Dioxynaphtalene, 2,7		"	$C_{10}H_6(OH)_2$	○○◒◍◍◍	○◉◉◉◉◉◉◉◉◉◉	○◉◉◉◉◉	vt	bk	k	●
Dinitrophenol		"	$C_6H_4(NO_2)_2$	○○○○○○	○○○○○○○○○○○	○○○○○○				⊖
Anilin		CTCl	$C_6H_5 \cdot NH_2$	◌●●●●●	●●●●●●●●●●●	●●●●●●	br			●
Aminophenol, m		H_2O	$OH \cdot C_6H_4 \cdot NH_2$	◌◍◉◉●●	○●●●●●●●●●●	○●●●●●	vt	vt	vt	●
Aminophenol, p		"	"	◉◉◉◉◉◉	●●●●●●●●●●●	●●●●●●	bk	bk	bk	●
Naphtylamine		CTCl	$C_{10}H_7 \cdot NH_2$	○◍◍◍◉◉	○◍◍◍◍◍◍◉◉◉◉	◍◉◉◉◉◉	vt	vt	vt	●
Aminobenzoic acid,p		H_2O	$\overset{COOH}{C_6H_4} \cdot NH_2$	○○◒⊕●●	○○○◒◒◌⊕⊕●●●	◌◍◉⊕⊕●	pk	pk	vt	●
Benzaldehyde	purss.	CTCl	$C_6H_5 \cdot CHO$	○◌◌◌○◌	○◒◌◌◌◌◌◌◌◌◍	○○○●●●	ye ↓br	br	bk	●
Benzoic acid	"	"	$C_6H_5 \cdot COOH$	○○○○○○	○○○○○○○○○○○	○○○○○○				⊖
Salicylic "	"	H_2O	$C_6H_4 \cdot OH \cdot COOH$	○○○○○○	○○○○○○○○○○◌	○◒◒◌◌◍	ye	gy	ye ↓bk	⊕
Gallic "	"	"	$C_6H_2(OH)_3 COOH$	◌◉◉◉◉◉	◉◉◉◉◉◉◉◉◉◉◉	◉◉◉◉◉◉	bl	bl	bl	●
Sulfosalicylic "	"	"	$\overset{SO_3H}{C_6H_3} OH COOH$	○○○○○○	○○○○○○○○○○○	○○○○○○				⊖
Tannin	pur.	"	—	○◒◒◌○◌	◒◌◌◌◌◌◌◌◌◍◍	○○○◍◍◉	bl	bl	bl	●
Xanthydrol	purss.	"		○○○○○○	○◒⊕⊕⊕⊕⊕●●●●	○⊕●●●●				●
Indole	"	"		○⊕●●●●	○●●●●●●●●●●	⊕●●●●●	bk	bk	bk	●
Tryptophane	"	"	—	○◒◒◍●●	○◌◍◍●●●●●●●	○◍◉◉◉◉	br	br	bk	●
Cyclohexanone	pur.	CTCl	$C_6H_{10}O$	○◌◌◉◉◉	○○○◒◌◍◉●●●●	○○●●●●	br	bk	bk	●
Campher, dl	purss.	"	—	○○○○○○	○○○○○○○○○○○	○○○●●●			bk	⊕
22Carotene	"	"	—	○◒◒◒◌◌	○◒◒◒◒◌◌●●●●	○○●●●●	br	br	br	●
Cholesterol	"	"	—	○○○○◒◒	○○◒◒◒◌◌◌◌◍◍	○◒◒◍◍◉	pk	pk ↓br	gy	●
Sodium-cholate	"	H_2O	—	○○○◌◌◌	○◒◌◌◌◌◌◌◌◌◌	○◌◌◌◌◌	vt	pk ↓bl	bl	
Sodium-taurocholate	"	"	—	○◒◒◌◌◍	○◒◒◌◌○○◉◉◉◉	○◍◍◉◉◉	br	br	br	

SUBSTANCE	PURITY	SOL.	FORMULA	0°C 0	1	2	16	24	48	20°C 0	1	2	3	4	5	7	16	20	24	48	50°C 0	1	2	16	24	48	COLOUR 0°	20°	50°	Ru
Lanolin	pract.	CTCl	——	O	O	O	▨	▨	▨	O	O	O	O	O	O	O	▨	▨	▨	▨	O	O	O	▨	▨	⊛	br	br	br ↓bk	⊛
Lecithin, animal	"	"	——	O	O	O	▨	▨	▨	O	O	▨	▨	▨	▨	▨	▨	▨	▨	▨	O	▨	▨	▨	▨	▨	br	br	br	⊛
23 Pyrrole	purss.	H_2O		O	▩	⊛	⊛	⊛	⊛	O	▩	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	⊛	O	⊛	⊛	⊛	⊛	⊛	bk	bk	bk	⊛
Proline	"	"		O	O	O	▩	▩	▩	O	O	O	▨	▨	▩	▩	▩	▩	▩	▩	O	▨	▩	▩	▩	▩	bk	bk	br	⊛
Oxyproline	"	"		O	O	O	▩	▩	▩	O	O	▩	▩	▩	▩	▩	▩	▩	▩	▩	O	▩	▩	▩	▩	▩	bk	bk	bk	⊛
Haemoglobin, haemolys.	—	"	——	aggl. ▨	▩	▩	▩	▩	▩	aggl. ▨	▩	▩	▩	▩	▩	▩	▩	▩	▩	▩	aggl. ▩	▩	▩	▩	▩	▩	br	br	br ↓bk	⊛
Chlorophyll	—	"	——	O	O	O	⊕	⊕	⊞	O	⊕	⊕	⊕	⊕	⊕	⊞	⊞	⊞	⊞	⊞	O	⊕	⊕	⊕	⊛	⊛				⊛
24 Penicillin	pract.	"	——	O	O	O	Ө	O	▩	O	Ө	Ө	Ө	O	O	O	▨	▨	▩	▩	O	Ө	O	▩	▩	▩	gy	bk	bk	
Aureomycin	"	"	——	O	O	O	O	O	▩	O	O	O	O	O	O	▨	▩	▩	▩	▩	O	O	O	▩	▩	▩	gr	br	gr ↓bk	
25 Carotene	pur.	CTCl	——	O	Ө	Ө	Ө	O	O	O	Ө	Ө	Ө	Ө	O	O	⊛	⊛	⊛	⊛	Ө	O	⊛	⊛	⊛	⊛	br	br	br	⊛
Aneurine	"	H_2O	——	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	Ө			gy	
Lactoflavine	purss.	"	——	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	Ө	Ө			or	
Ascorbic acid	"	"	——	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	▨	▩	▩	br	br	br	⊛
Nicotin amide	"	"	——	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O				⊛
26 Pilocarpine	purss.	H_2O	——	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O				⊛
Colchicine	"	"	——	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	Ө	Ө	O	O	Ө	O	O	O		gy	gy	⊛
Brucine	"	"	——	O	O	O	Ө	Ө	▨	O	Ө	Ө	Ө	Ө	O	▨	▨	▨	▨	▨	O	Ө	O	▨	▨	▨	br	br	bk	⊛
Strychnine	"	"	——	O	O	O	▨	▨	▨	O	O	O	O	O	O	O	▨	▨	▨	▨	O	▨	▨	⊕	⊞	⊞	pk	pk	pk	⊛
Ergotamine	"	"	——	O	O	O	⊛	⊛	⊛	O	▨	▨	▨	▨	▨	▩	⊛	⊛	⊛	⊛	O	▨	▨	⊛	⊛	⊛	vt	br	bk	⊛

Explanation of Symbols and Signs

Purity

purss. = purissimum, analytical quality
pur. = purum, c.p. high grade purity
pract. = practicum, technical product

Colours

bk = black	or = orange
bl = blue	pk = pink
br = brown	rd = red
gr = green	vt = violet
gy = grey	ye = yellow

O	No reaction
ӨӨӨӨ	The reaction consists of a colour development
▨	weak reaction } OsO₄ is reduced to a colloidal form of lower oxydes
▩	strong " }
⊕	Substance and osmium
⊞	precipitate
⊛	in different strength
aggl. O	Agglutination, Coagulation, Precipitation
⊕ ⊞ ⊛	Ruthenium reacts with different intensities
⊖	" exhibits no reaction

of which may be lower oxides (51, 49), others esters or complexes of the tetroxide. Osmium tetroxide is considered to be the only possible colourless osmium compound concerned here (38). In the tables ↓ indicates a change in colour between the two colours listed for one temperature. For substances with low but measurable solubility saturated solutions have been used. Some substances are insoluble in both water and carbontetrachloride. Their reactions are then judged according to the colour change on their surface, i.e. a blackening.

Some preparations are listed twice according to their biological relationship and from a more chemical point of view. The purity of the preparations used is indicated in the third row (see explanation of symbols and signs). The last row gives roughly the results of the reactions between *ruthenium tetroxide* and the listed substance at 20° C in similar proportions as for osmium tetroxide; crosses indicate reactions. The reaction readings are divided into two parts: on the *left side* of the table 0° C: 0–48 hrs and 20° C: 0–7 hrs, which represent the most interesting conditions for electron microscopy, and on the *right side* of the table longer reaction times and higher temperature, which may be of principal interest for the reaction studied.

Physical data for OsO_4 that serve to give a better understanding of its mode of reaction:

Electrical conductivity less than 10^{-11} reciprocal Ohm (this means the substance can be regarded as non-ionized (64)).

Solubility in water, 25° C	7.24 g ± 0.01 g/100 ccm,
Solubility in carbontetrachloride, 25° C . . .	375 g ± 17.0 g/100 ccm (2).

It is said to be an unsaturated coordination compound (33, 35), which is in accordance with calculation from the parachor (61). The four oxygen atoms are paired and each pair is at a different distance from the central atom (11). This is not quite in agreement with the regular tetrahedral arrangement of the OsO_4 molecule postulated by Langseth and Oviller (34). The dipole moment is = 0 (11). It can be shown that OsO_4 dissolves slowly but readily in saturated lipids, paraffin oil and palmitic acid.

DISCUSSION

Comments on the Tables

1. *Saturated hydrocarbons* react very slowly or not at all. The pronounced reactivity of the double and triple bond is demonstrated. The reaction of propane at 50° C is due to impurities. Paraffin wax is known often to contain cyclic compounds, even unsaturated ones, and the latter may explain the observed reaction. The brown colour at 50° C is likely to indicate reactions with unsaturated bonds. That acetylene is attacked by osmium tetroxide has been reported earlier (30, 37). In the Criegee reaction 1 molecule of acetylene reacts with 2 molecules of osmium tetroxide (20).

2. *The halogen derivatives* show no reactions and can therefore be used as solvents for OsO_4.

3. *Alcohols and ethers.* The hydroxyl group shows its maximum reac-

tivity attached to carbon chains of 3–5 carbon atoms and decreasing reactivity towards longer chains, e.g. cetyl alcohol. Heptyl alcohol was a sample of lower grade purity.

The unsaturated alcohols exhibit a reactivity that increases with the number of double bonds in the carbon chain but decreases with increasing chain length. Brown is again a favoured colour for reactions of double bonds with OsO_4. An increase in the number of hydroxyl groups or an ether configuration does not increase the reactivity towards osmium tetroxide.

The quick reduction of the osmium tetroxide by ethanol was observed in the very early days of the discovery of this metal (62, 63); aldehydes and acids are formed (10).

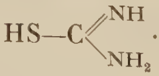
4. *Compounds containing —SH or —S—.* These react immediately with OsO_4 (thioglucollic acid, glutathione). Cysteine reacts fast when alkalized. The reaction of its hydrochloride exhibits some similarity to the reaction of cystine, indicating that these reactions may be essentially the same. Cysteine is oxydized in a first step. After addition of one drop of OsO_4 solution it no longer gives a nitroprusside reaction. In a second step—as with cystine—sulfonic groups are established which can be demonstrated in the supernatant.

These sulfhydryl and disulfide reactions are in good agreement with the very quick action of osmium tetroxide on methyl-hydrosulfide or sulfide (52).

5. *Amines.* Generally it can be said that a pronounced affinity of the tetroxide for nitrogen compounds is demonstrated in this series. Amino groups exhibit increased reactivity when attached to longer carbon chains. Methylamine→Heptylamine. Hydrazin is known to be a strong reducing agent and in the literature hydrazin sulphate is reported to be oxydized by OsO_4 to N_2 (30).

6. Neither *aldehydes* nor *ketones* exhibit a pronounced reactivity, although an aldehyde group on longer chains shows a reaction. Acetylacetone reacts probably on account of the double bond of the enol form or the two labile hydrogen atoms.

Shorter aldehydes and saturated ketones possess little tendency to react (30).

7. *Carbamic acid derivatives.* This series demonstrates the reactivity of the imino group in guanidine and again the role of an unoxydized sulfur atom towards osmium tetroxide. The very intense reaction of thiourea can also be explained by its tautomeric form  $HS{-}C\begin{smallmatrix}\nearrow NH \\ \searrow NH_2\end{smallmatrix}$. The two amino groups do not seem to contribute to the reactions.

8. *Amino alcohols and derivatives.* The reaction of ethanolamine resembles that of ethylamine, somewhat weakened by the presence of a hydroxyl group. Cholin, as well as triethanolamine, forms a coordination compound, but this is not possible when the nitrogen in cholinchloride is occupied by chlorine. For coordination compounds with OsO_4 see group 20.

9. *Esters* show practically no reactions.

10. *Greases and oils.* The reactions of greases with "osmic acid" have been known since the early days of the use of osmium tetroxide. The only interesting feature of this series is the fact that a great number of greases and oils do not form precipitates. They repeat the reactions of compounds with double bonds, because all of them contain unsaturated fatty acids in varying proportions. Some other substances, at least in coconut fat, lard and butter, may contribute to the reactions.

That osmium tetroxide furnishes a specific means of detecting fats in general has often been doubted (28, 46, 29, 48, 36). Altmann (1) was the first to show that only unsaturated fatty acids react with OsO_4, which was further verified with purified substances (26, 45).

The specific way in which osmium tetroxide attacks certain double bonds has subsequently been made the subject of many studies. One approach to this was the study of Hofmann and co-workers (31, 30). Olefinic double bonds are attacked by OsO_4, forming diols, and if the reaction is performed in a solution of potassium chlorate the reduced tetroxide is immediately reconstituted. Thus the tetroxide works as a catalyst. This procedure was subsequently used in a wide field of diol preparations and double bond studies in unsaturated fatty acids (40, 3), in cyclic olefines (18) and for the oxydation of fumaric and maleic acid (67). Thus the inertness of formic, acetic and oxalic acid towards this procedure was reported (30). A further approach introduced by Criegee (19, 20) is the application of the osmium oxydation to aromatic hydrocarbons in the presence of a tertiary ammonium base. Osmium tetroxide and pyridine form a coordination compound that easily reacts with certain double bonds in cyclic compounds establishing an ester. The ester is a rather stable compound. Upon hydrolysis it yields a cis-diol in a stoichiometric reaction. OsO_4 alone is also able to form, in a relatively slow reaction, monoesters that are black-brown but rather liable to an autooxidative breakdown.

The presence of tertiary bases accelerates the reactions appreciably. With anthracene and other polycyclic compounds osmium tetroxide does not attack the very active meso-positions but acts exclusively on the 1 : 2 (20) and the 3 : 4 positions (17, 44). These positions are the "K" positions of Pullmann (56), suggesting that similar electronic conditions are the principal basis for the double bond reactions of osmium tetroxide in general. The Criegee reactions for double bonds are a means of determining the relative reactivity of such bonds (4, 5, 13). Certain groups in the molecule, e.g. the methyl group, may favour the reaction, while others, e.g. the cyano group, have the reverse effect.

11. *Monocarboxylic acids* show low reactivity. The unsaturated acids again exhibit the features of double bond reactions.

12. *Oxycarboxylic acids.* The reactions of these biologically important acids seem to be dependent on the oxidation of the α-carbon. Lactic acid is said to be oxidized to aldehyde and CO_2 (30).

13. *Dicarboxylic and tricarboxylic acids* do not react, whether their carbon chains are oxidized or not. Odd and even carbon numbers differ somewhat in reactivity.

The inert behaviour of dicarboxylic acids has its explanation in the fact that some of these compounds can be synthesized by means of osmium tetroxide oxidation. Cyclohexanone→adipic acid, fumaric and maleic acid→tartaric acid (41).

14. In line with the behaviour of the dicarboxylic acids is the behaviour of the *carbohydrates.* Neither the single pentoses or hexoses nor their polymers react. Lignin was a very carefully prepared sample, kindly supplied by Dr. Gierer, Swedish Forest Products Research Laboratory, Stockholm. Phosphorylation of the carbohydrates does not alter their reactions substantially.

The very slow oxidation of cane-sugar to oxalic acid was reported earlier. Gum arabic, mannit and starch did not show any reaction within two days (14).

15. *Amino acids* (cystine, tyrosine and diiodotyrosine in saturated, the others in 0.01 molar solutions). The amino acids and their related compounds can be grouped according to their reactions into 6 types:

(i) *a*, the α-amino acids without reactivity: glycine, alanine, aminobutyric acid, valine, norvaline, leucine, isoleucine, norleucine. *b*, phenylalanine, tyrosine, diiodotyrosine, hippuric acid. *c*, and with somewhat increased reactivity: serine, aspartic acid, threonine.

(ii) betaine, creatine, creatinine. These compounds are marked by their striking inertness towards OsO_4. It seems justifiable to assume that this is brought about by the inner salt formation in these compounds. This view stresses again the role of amino groups. Thus in group:

(iii) Diamino acids, the second amino group is responsible for the reactivity towards the tetroxide: *a*, ornithine, lysine, *b*, asparagine and glutamine (possibly some steric hindrance). The reactions of hydrochlorides of these compounds support this view.

(iv) Arginine resembles the reaction of guanidine, while citrulline is similar to that of urea. For arginine again the role of the "free" amino group is obvious. The reaction of sarcosine reminds of that of diethylamine.

(v) In cysteine, cystine and methionine, as already described in group 4, the unoxidized sulfur possesses a strong affinity towards OsO_4 and causes the reactions listed.

(vi) Amino acids consisting of cyclic compounds and alanine show only the reactions of their cyclic groups. The alanine residue does not take part in the reaction. Thus dioxyphenylalanine gives the reaction of pyrogallol, tryptophane the reaction of indole, histidine of pyrimidine (the dihydrochloride probably reacts through its double bond), proline and oxyproline exhibit the reaction of pyrrole.

16. *Peptides*. The behaviour of the amino acids towards osmium tetroxide may be expected to be similar when they are bound to each other by peptide bonds. The results show no significant reactivity difference, although one could assume the slight increase in reactivity to come from terminal amino groups. Glycyl-tryptophane resembles the reactive indole. When the strongly reducing sulfhydryl group in glutathione is oxidized with oxygen peroxide it does not react any longer. Ergotamine and penicillin are listed because on modern views they count as peptides (12).

17. *Proteins* were tested in 5 per cent solutions, and in some cases saline was added until the protein was completely dissolved. The volume of reagent was reduced when expensive substances were used. If nothing else is indicated the preparations were commercially "purified". Globin, horse blood, is an older preparation. When substances are listed twice they are from different manufacturers.

Because so little is known about the exact structure of proteins and in particular their group reactivity (e.g. masked and free —SH groups), an interpretation of the mode of reaction for each substance will be difficult. Nevertheless it may be justifiable to suggest an estimate of the reactivity of a protein towards osmium tetroxide roughly from its tryptophane, cysteine and histidine/2 content, which corresponds fairly well with most of the data reported here.

Incorporated in large molecules arginine and lysine do not seem to give an increase in reactivity, even when occurring in large amount (protamine: 85 per cent N as arginine).

18. It seems still more difficult to give an interpretation of the enzyme reactions with OsO_4. Applying the above rule of summing the tryptophane, cysteine and histidine/2 content one would predict a pronounced reactivity for ribonuclease and lecithinase. Phosphorylase could not be freed perfectly from the stabilizing cysteine, but trypsin and pepsin follow the proposed rule.

19. Most interesting is the inertness of the *nucleic acids* towards osmium tetroxide. The manufacturers are: RNA I Hoffmann la Roche; RNA II German Merck; DNA I, low polymer, Schwarz Laboratories; DNA II, high polymer, Worthington; Na-nucleinate Lemke & Co., N.Y.

20. From the reactions of the nucleic acid constituents the inertness of the whole nucleic acid compounds can easily be understood. Uric acid is an exception and its behaviour is independent of the origin of the preparation. I and II are from different sources but reacted similarly. The reaction of II listed here was performed in alkaline solution.

Pyridine and other tertiary bases such as picoline and quinoline interact quickly with OsO_4 to form a complex (see also indene and triethanolamine group 8). The coordination compound consists of one or two molecules of pyridine per OsO_4 (16, 20, 4). Such a complex is rather stable and increases the general liability of OsO_4 to ester formation. Interactions with proteins or nucleic acids and osmium tetroxide have been studied repeatedly (24, 22, 43, 39, 27, 9, 32, 8, 55). The summarized results of these earlier studies and our own observations are as follows: There is no obvious interaction with isolated nucleic acids, whether the osmium tetroxide acts upon these substances in an acid, a neutral or an alkaline solution in the time and under the temperature conditions used for histological and electron microscopical work.

Not all reacting proteins reduce the tetroxide directly to a lower oxide. The reaction proceeds in most cases slowly, comprising alterations of the viscosity, the optical properties, heat coagulability, alcoholic coagulability, and the staining properties. This holds true mainly for concentrated solutions. Diluted protein solutions do not seem to be affected by the addition of osmium tetroxide. A prolonged action of the tetroxide on proteins can result in a liquefaction.

A decrease in pH values for tissues and protein solutions during OsO_4 action has been observed (55, 50), which is probably effected by an elimination of basic groups (e.g. tryptophane), the reduction of OsO_4 to an acid residue OsO_3 (which has no free existence), and the oxidation of certain groups.

21. *Aromatic hydrocarbons.* Simple cyclic compounds exhibit about the same reactivity as the paraffins. Anthracene reacts according to the Criegee reaction. Halogenation of the aromatic rings does not markedly increase their reaction, neither do single hydroxyl groups in phenol, thymol and naphtol (compare the reactions of phenylalanine, tyrosine and diiodotyrosine). The reactions of cyclic hydrocarbons with more hydroxyl groups correspond directly to the reaction of dioxyphenylalanine. Nitro groups are without influence, but the aromatic amines show intense reactions at their amino groups, a significant feature of this group already demonstrated in previous sections. Benzaldehyde, like oenanthol, illustrates the role that an aldehyde group may play under suitable conditions in interactions with osmium tetroxide.

The reactions of the next substances in the table support the above conclusions and could have been predicted. Indole is of special interest because of its relation to tryptophane, for which it obviously represents the reacting part. It may react with the double bond between the α and β position (47) or with its nitrogen participating in a coordination compound with OsO_4. Like iron ions OsO_4 gives violet or blue colorations with pyrogallol, gallic acid, gallotannin and catechol (62, 14, 30, 42), indicating that three hydroxyl groups in juxtaposition are prerequisites for a permanent colour reaction. Reactions of this type assume importance when plant material, containing tanning substances, is concerned.

22. Carotene, as well as lanolin, react with their unsaturated side chains, lanolin probably with its basic sterol structure. Cholesterol and cholates exhibit the reactions described by Criegee which have been applied repeatedly in hormone studies and preparations (for ref. see (20)). It was observed that kephalin and lecithin preparations quickly react with OsO_4 (25, 65, 29). This is probably a double effect of both unsaturated fatty acids and the choline or colamine residue in their molecule. Quite apart from such considerations the rate of osmium reduction in the adrenal cortex of the rat upon stimulation has been regarded to be a direct indication of organ activity (46, 23). Our own studies support this view, giving significant values for the osmium uptake in different tissues. The results will be published later.

23. The *pyrrole* ring shows a strong reaction; even in its saturated form, the pyrrolidin, in proline and oxyproline, there is still pronounced reactivity, but incorporated in a protein molecule these two compounds do not react with OsO_4 (gelatin). In haemoglobin and chlorophyll pyrrol rings might contribute to the reactions.

24. The antibiotics as well as the alkaloids are listed to demonstrate the

reactions of complicated compounds whose mode of reaction is difficult to interpret.

25. *Vitamins.* For carotene see 22, above. Ascorbic acid is known to be a reducing agent as a result of its two enol groups. The inertness of nicotinamide is not quite understood although some parallel exists in the reaction of urea.

26. See group 24.

Ruthenium Tetroxide

The unique relationship between osmium tetroxide and ruthenium tetroxide led us to investigate the possibilities of an application of the latter substance as a fixative. This has been studied previously (57, 15) and certain advantages were claimed for it, but these advantages could not be substantiated in our study. As expected from the pronounced reactivity and lability of the compound an attempt to fix tissue pieces (liver, intestine and muscle) in a 1 per cent solution at 0° C resulted in a fast precipitation of all ruthenium as a black deposit. The tissues showed only slight changes on their surfaces. The reactions of the ruthenium tetroxide listed in the last column of the tables serve to elucidate the general behaviour of this compound towards organic substances.

In connection with this study about the reactions of ruthenium tetroxide it may be of interest to note that an attempt to apply *ruthenium-red* as an electron stain for connective tissue resulted in total destruction of all tissues during methacrylate embedding procedure.

SUMMARY

Few observations giving precise information about the reactions of osmium tetroxide with biologically important substances exist in the literature. In spite of this fact the general features of such reactions have been recognized. The most reliable information is found in the chemical literature, at least about the osmium tetroxide reactions with double bonds. These data and the results of our own study are derived from reactions under relatively well known *in vitro* conditions and cannot be transferred directly to a cellular system. Nevertheless such studies may give some idea of the possible reactions and help in interpreting the structures seen in ultrathin sections of osmium fixed material, where the osmium deposits are about the only "scattering" constituents.

An approximate order of reactivity is suggested: *Sulfhydryl groups* have a direct reducing action, which may partly account for reactions with e.g. peptides, proteins and enzymes. *Double bonds* show about the same reac-

tivity, an outstanding property of osmium tetroxide. Reactions with olefinic chains are exothermic and fast, but the esters formed are rather liable to hydrolyse into a diol and $OsO_4 \cdot 2H_2O$. In ester formation with cyclic compounds the osmium tetroxide is firmly bound to certain double bond positions in the molecule concerned and gives rather stable products. This reaction type is of interest for fats, waxes, lecithin, the cerebrosides, vitamins, certain hormones, gallic acids and all other biological substances having a basic sterol structure. With tertiary bases the osmium tetroxide molecule easily forms stable coordination compounds, which in turn are able to accelerate the double bond reactions appreciably. Such coordination probably occurs with e.g. *tryptophane*. *Amino groups* in a terminal position and not salt linked, *sulfide sulfur, hydroxyl and aldehyde groups* in terminal positions and on certain carbon chain lengths, certain *heterocyclic compounds* and *aromatic compounds with hydroxyl groups in juxtaposition* are able to give intense reactions with osmium tetroxide, the latter being of importance when plant material containing tanning substances is studied.

Criegee, and the workers using his method, showed that a large number of the osmium tetroxide reactions with organic substances are affected by steric conditions. This fact explains in some cases the inertness of one and the reactivity of another compound both having very similar structures.

The inertness of the *carbohydrates* and the *nucleic acids* towards OsO_4 is one of the most striking results obtained and may be an explanation of the nonimpregnated areas in cytoplasm and nucleus in ultrathin sections of osmium fixed tissues.

From empirical considerations it is suggested that *protein* reactions with osmium tetroxide are roughly dependent on the tryptophane, cysteine and histidine/2 content in the protein. Some enzymes obviously form exceptions to the rule.

Even though a rough impression of the sites of reaction of osmium tetroxide and thus a vague interpretation of the osmium deposits in sections can be given, no conclusions can be drawn from the localisation of the osmium reactions in the heterogeneous cellular structures. The reactions of osmium tetroxide with biological material are dependent on time, temperature and concentration, and thus the results in the literature and our own studies suggest that *constancy of time, temperature, object size, and composition of the fluid are essential to get comparable results when osmium tetroxide is used for fixation.*

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STUDIES ON THE EFFECT OF X-RAYS ON THE BIOCHEMISTRY AND CELLULAR COMPOSITION OF ASCITES TUMORS

II. CHANGES IN THE PATTERN OF GLYCINE-2-^{14}C INCORPORATION DURING THE FIRST TWO HOURS AFTER IRRADIATION *IN VIVO*

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A previous communication reported studies concerning the effect of X-rays on the following characteristics of the Ehrlich ascites tumor: growth rate, mitotic index, average cell volume and total amount of pentose nucleic acid (PNA), desoxypentose nucleic acid (DNA), and nitrogen per cell (14).

An *in vivo* dose of 1250 r was found to inhibit mitotic activity completely during a period of 16–20 hours. Division figures reappeared thereafter and reached almost normal values 48 hours after irradiation. Many of these division figures were abnormal. The great majority of the cell population remained viable during this whole period, as judged by supravital staining.

There was a progressive increase of cell volume during the amitotic period following irradiation. Total N and PNA increased at about the same rate as the volume, their average intracellular concentration thus remaining unaltered. By contrast, there was only a very slight and insignificant increase of the amount of DNA per cell, indicating a specific inhibitory effect of irradiation on DNA synthesis.

Using the same biological material and the same dose of X-rays, the present investigation is concerned with changes in the incorporation pattern of glycine-2-^{14}C into a number of biochemical fractions during the earliest stage after irradiation (35–150 min.). During this short time the changes in cell volume and composition are small and the present results, calculated per unit weight, reflect therefore the changes per average cell.

MATERIALS AND METHODS

Mice.—Hybrid albino mice, received from a commercial farm, were used. All experiments were carried out with 3–4 months old males with a body weight of 20–

25 g. The animals were kept on a standard compressed diet which, as well as drinking water, was available *ad libitum*.

Tumor.—The Ehrlich ascites tumor was used in the following way: Ascites was collected from animals inoculated 6–14 days earlier. The fluid was stored in the refrigerator and the concentration of cells has been counted in the meantine in a Bürker hemocytometer. The proportion of tumor cells was established by means of differential counts on stained smears. Dilutions of the fluid were then made with buffered physiological saline and 1.8×10^6 tumor cells were injected intraperitoneally to groups of 80–90 mice in volumes of 0.2–0.5 ml.

In order to study the possible differences in the effect of irradiation on tumor cells of different physiological age and growth rate, animals were sacrificed at various times after inoculation (Table I). Group 1 contains 163×10^6 free tumor cells per mouse on an average, growing at a relative rate of 2.12 per cent per hour. The corresponding figures for group 2 were: 466×10^6 cells and a relative growth rate of 1.50 per cent. Group 3 contained 845×10^6 cells with the relative growth rate 1.22 per cent. The total number of tumor cells and their relative growth rate was calculated as described previously (15). The tumor cells grow under rather anaerobic conditions (17) and the degree of anaerobiosis probably increases with the physiological age of the tumor. Simultaneously there is an increasing shortage of nutrients as cell multiplication proceeds. Biochemical changes which are functions of the physiological age of the tumor cells have to be considered primarily against the background of these changes in cell nutrition and O_2 supply.

In each group, half of the animals were irradiated and immediately thereafter the whole group was injected with the isotope which was administered alternately to irradiated and control mice. They were killed 35, 60, 80 and 100 minutes after the isotope injection. Their ascites was collected, pooled and analyzed as described below. In some cases, groups of four mice were also killed 120 and 150 minutes after isotope administration. The relevant analyses were pooled and plotted as the average value for 135 min.

Irradiation.—The mice were irradiated over the abdomen with 1250 r at 165 kV, filtered by 1 mm Al, and at an intensity of 1300 r/m.

Isotope technique.—Glycine-2-^{14}C, dissolved in saline at pH 7.0, was injected intraperitoneally in amounts of 0.5 μC/0.1 ml saline per mouse with an Agla Micrometer Syringe. A uniform distribution in the ascitic fluid can be expected within a short period of time because of the continuous mixing of the fluid due to the movements of the mouse and to intestinal peristalsis. Experiments with dye-injection (22) showed uniform distribution within five minutes through the entire ascites.

To assay the activity in the small amounts of isolated compounds it was necessary to apply the "infinite thin layer" technique. 0.2 ml amounts of the dissolved compounds measured by the Agla-syringe, were desiccated on Al plates and with an area of 3 square cm covered by linse paper. Two or more parallel determinations were performed. The concentration range within which a linear relationship holds for the number of counts per minute and the amount of substance was determined in separate trials. In the present work identical amounts of substance within the linear range were poured on the plates. Duplicate measurements agreed usually within 3 per cent; if this was not the case the measurements were repeated on new plates. The G-M tube was thoroughly shielded with old lead and gave a low background. Nevertheless, the low activity made it sometimes necessary to continue counting up to 20 hours with certain plates.

Chemical procedure.—Ascitic fluid was collected in ice-cooled centrifuge tubes at the time intervals stated above. Cells and ascitic serum were separated by centrifugation. Small samples of the thoroughly mixed sediment and of the supernatant were dried at 60° C, powdered and dissolved in 0.02-*n* NaOH. After dilution with distilled water, aliquots were taken to determine the total activity per unit weight for the cells and for the serum.

The main bulk of the cellular sediment was extracted twice with 1.5 vol. of ice-cooled 10 per cent and 5 per cent solutions, respectively, of trichloroacetic acid (TCA). The pooled extract was separated into a "Ba-insoluble" and a "Ba-soluble" fraction by precipitation with Ba-acetate at pH 6.8, following the procedure of Le Page (16). The precipitate contains orthophosphate, ATP, ADP, hexosediphosphate and phosphoglyceric acid. All the organic compounds can be expected to have incorporated ^{14}C. The Ba-soluble fraction contains, among others, free glycine-^{14}C as a contributor to the activity, especially soon after irradiation. To make the absorption by salt negligible the activity of this fraction was measured after a three-fold dilution with water and was calculated to represent the activity obtained by extracting one gram of the wet cellular sediment.

The Ba-precipitate was dissolved in 0.5-*n* HCl, and, after having removed Ba with Na_2SO_4, the supernatant was evaporated to dryness *in vacuo*. The remainder was dissolved in distilled water and the HCl removed by repeated evaporations. The activity of this unfractionated Ba-free precipitate per unit weight was determined. Thereafter it was hydrolyzed with 1-*n* H_2SO_4 in a boiling water bath for one hour. Adenine was precipitated with $AgNO_3$, extracted from the precipitate with acid and reprecipitated. The UV-extinction of the extracted free adenine was measured and identical amounts were plated for activity determinations. The results were expressed as number of counts/min/mg adenine.

In a few cases attempts were made to determine the relative proportions of ATP and ADP in the Ba-precipitate by chromatography according to Cohn (5). It was found to contain mainly ATP. However, as the proportions of ATP to ADP may vary with the physiological age of the cells, and after irradiation, this fraction was denoted as ATP-ADP. The total amount of ATP-ADP adenine per cellular wet weight, as well as its spec. act., were calculated. Subtracting this activity from the value for the total Ba-precipitate, the spec. act. of the heterogeneous residue was obtained and designed as "Ba-free residue".

The residue after the TCA-extraction was washed with cold distilled water and extracted twice with a boiling 3:1 mixture of ethanol and ether. The pooled extract was evaporated at room temperature and the remainder dissolved in petrol-ether. The material obtained after an additional evaporation and dissolution in petrol-ether was designed "Total lipids". No further fractionation was carried out on this material.

The nucleic acids and proteins were isolated from the extracted residue as Ba-salts, according to the method of Le Page and Heidelberger (18). The nucleic acids obtained are impure, being contaminated mainly with proteins. The main part of the activity in the nucleic acids is due to incorporation of ^{14}C into the purines, however. As the amount of available material was rather small, no further purification of the nucleic acids was carried out but the purines were isolated as Ag-salts and purified. Identical amounts of free purines (as calculated from the extinction data) were plated for counting. There were made no separate analyses of the proportions of adenine and guanine and the results were expressed as counts/min/mg purine.

After separation from DNA the proteins were purified by extraction with 5 per cent TCA at 90° which was continued until the extract did not exhibit any specific adsorption at 2600 Å. The residue was hydrolyzed in 0.05 *n* NaOH, diluted with distilled water to make salt absorption negligible and measured for activity.

RESULTS

General distribution of activity.—Table I shows the distribution of ^{14}C in the control and X-rayed material. The values refer to the four time intervals 35, 60, 80 and 100 minutes after injection of the isotope. Group 1 consists of the average of the results of four different samples, each of which was obtained by pooling the ascites of four mice. Group 2 represents the pooled results of twelve samples, and group 3 those of eight samples. The main data obtained for the various biochemical fractions studied are given in cols. 4–8, Table I, with the exception of the "Ba-free residue" and the lipids which contain but small part of the total cell activity and are of a complex and unknown composition. Cols. 9–13 show the ratios between the spec. act. of

TABLE I

Distribution of ^{14}C in various biochemical fractions of the tumor cells. Pooled values of all analyses performed 35, 60, 80 and 100 minutes after irradiation.

	1	2	3	4	5	6	7	8	9	10	11	12	13
			Counts per min. per mg of:						Ratio of cols.:				
oup	Cell-weight, g per 4 mice	Total counts per min. × 10^4	Wet cells	Proteins	DNA	PNA	ATP-ADP	Ba-soluble from 1 mg cells	$\frac{4}{3}$	$\frac{5}{3}$	$\frac{6}{3}$	$\frac{7}{3}$	$\frac{8}{3}$
						Controls							
1	0.9	8.7	96.5	293	882	1,310	11,300	21.9	3.05	9.15	13.5	117	0.226
2	3.0	13.5	45.2	91.7	188	498	5,170	10.0	2.03	4.16	11.0	114	0.221
3	5.2	12.6	24.1	43.1	79.5	325	2,980	8.4	1.79	3.30	13.5	123	0.348
						X-rayed							
1	1.1	9.5	86.5	232	572	895	9,750	24.2	2.68	6.61	10.4	113	0.280
2	2.7	13.1	48.5	92.0	130	371	5,000	11.3	1.90	2.68	7.65	103	0.234
3	5.2	12.2	23.5	40.6	60.6	216	2,660	7.7	1.73	2.58	9.19	113	0.327

the various fractions and that of the whole cells (col. 3), giving information on the distribution of ^{14}C between the various fractions.

In spite of great differences in the average cell weight obtained from the three groups with different physiological age (col. 1), the total activity shows but small variations (col. 2). The slightly but consistently lower activity in the cells of group 1 as compared to groups 2 and 3 is probably due to the fact that a comparatively larger amount of labeled glycine is resorbed from the peritoneal fluid during the interval between injection of the isotope and collection of ascites if the volume of the fluid is small (22).

The decreasing cellular spec. act. (col. 3, Table I) with increasing cell age is due to a dilution of the injected isotope. The spec. act. of controls and X-rayed cells is reasonably similar for each group, however. This is of particular importance and will be discussed later in connection with the measurements of activity in the various biochemical fractions and the distribution of activity within the cell.

Ba-soluble fraction.—The results are shown in col. 8, Table I. In col. 13, the ratios between the spec. act. of the Ba-soluble fraction and that of the whole cells are given. It appears that the ratio is highest for the physiologically oldest cells of group 3. The activity is partly due to free glycine and partly to low molecular intermediates in the metabolism. As will be shown below, physiologically old cells incorporate less isotope into some high-molecular cellular compounds than younger ones, which is the probable explanation of the higher activity in their Ba-soluble fraction. The differences between group 1 and 2 (col. 13) are of doubtful significance, as only four determinations were made with group 1.

The Ba-insoluble fraction.—Treatment of the TCA-extract with Ba-acetate resulted in the precipitation of 1.66 mg substance per g wet cell weight in the control and 1.86 mg in the irradiated samples on an average. The difference is not significant statistically but suggests a higher concentration in the irradiated cells. This will be amplified by the results on the ATP-ADP fraction.

a) *ATP-ADP.* Fig. 1 shows the changes in the total amount and spec. act. of this fraction with time in X-rayed cells as compared with the controls. As there was no essential difference between cells with different physiological age, the values from all the groups were pooled.

It appears that the amount of ATP and ADP per cell shows a considerable but transient increase soon after irradiation. The surplus amount disappears at a high rate and in an approximately linear fashion between 35 and 100 minutes after irradiation. A test for the significance of the correlation gives

$r = 0.846$ and $0.01 > P > 0.001$. The value at 135 minutes suggests a second increase and possible cyclic changes in this system after X-ray treatment.

The incorporation of ^{14}C into ATP-ADP has to be considered in relationship to the changes in the amount of this fraction. Corresponding to the increase in amount there is a decrease in the spec. act. of ATP-ADP soon after irradiation. This probably means that the synthesis of ATP-ADP proceeds at a fairly normal rate after irradiation while its consumption is inhibited. The incorporation of glycine-^{14}C into ATP-ADP proceeds at a rate limited by diffusion processes and by the time necessary for the formation of precursors. The ^{14}C-labeled ATP-ADP formed during the first 35 minutes after irradiation would therefore be diluted by the higher concentration of inactive ATP-ADP in the cells.

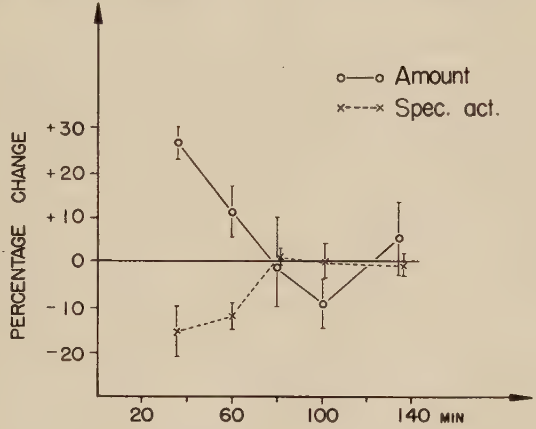

Fig. 1. Percentage change in the amount and spec. act. of ATP-ADP in irradiated cells compared with controls.

b) *The "Ba-free residue"*. This heterogeneous fraction contains orthophosphate, glycerophosphates, and hexosephosphates. Its "spec. act." is shown in Table II.

TABLE II

"Spec. act." (counts/min/100 γ) of the Ba-free residue. Each figure is the mean of six detns. during the period 35–100 min after irradiation.

Group	Control	X-rayed
1	60.7	30.9
2	21.9	21.7
3	11.2	7.2

The incorporation rate in cells of different physiological age follows rather closely the spec. act. of the total cells. Irradiation depresses the incorporation of the isotope by 35 per cent on an average. The X-ray effect appears to be more pronounced in the youngest cells of group 1, although the size of the material does not permit definite conclusions. In the absence of detailed analyses the possibility remains that the effect of X-rays on the activity is due to changes in the amount of one or several compounds within the fraction rather than to variations in spec. act.

Nucleic acids.—Incorporation of ^{14}C into the purines of PNA and DNA was less in the irradiated mice than in the controls. The experimental material (Table III) indicates that the average effect is of a similar order for DNA as for PNA. The depression is manifest already 35 minutes after irradiation and it seems to be more intense at the two extremes of the observation period, 35 and 135 minutes, than during the intermediate time interval. A similar tendency was found in the lipid fraction. The figures for DNA suggest that the effect on old cells (group 3) may be slightly less pronounced during the first 100 min., probably as a consequence of the low metabolic rate.

Proteins.—The X-ray effect is slight and seems to be rapidly reversed after an initial depression (Table I and Fig. 2). The relative stability of the proteins against irradiation found here is in agreement with earlier investigations as discussed below.

Lipids.—The total amount of lipids was not altered by the irradiation, being 12.6 mg/g wet cellweight on an average. Physiologically older cells contained somewhat higher amounts of lipids than young ones. Incorpora-

TABLE III

Percentage change in the incorporation of ^{14}C into the nucleic acids after irradiation.

Time after irradiation min.	PNA				DNA			
	Group			Mean	Group			Mea
	1	2	3		1	2	3	
35	-25.6	-42.5	-35.1	-37.2	-40.0	-38.9	- 6.2	-28
60	-50.4	-18.6	-26.7	-26.6	-27.5	-10.7	+12.6	- 5
80	-27.2	-15.5	-28.3	-21.7	-31.6	- 8.5	-21.2	-16
100	-22.7	-22.5	-25.0	-23.4	-37.9	-30.8	-30.0	-31
(135)	-50.8	+15.4	-59.2	-31.5	-37.5	-38.7	-58.5	-44
Mean	-31.5 ± 6.38	-24.8 ± 6.63	-28.8 ± 1.8	-28.1	-34.2 ± 2.88	-22.3 ± 6.79	-11.1 ± 9.02	-25

tion of ^{14}C resulted in a spec. act. of the order of 5–10 counts/min/mg in the controls. Rapid changes in the turnover rate of one or several fractions in the complex mixture of lipids characterize the effect of X-rays on the incorporation of ^{14}C (Fig. 2).

Time-effect relationships.—The spec. act. of the ascites serum decreases gradually after the isotope injections at approximately the same rate in X-rayed and control samples. The activity at 100 min. is only one fourth of that at 35 min. This is partly due to an uptake of labeled glycine by the tumor cells and partly to a resorption by the host tissues. The spec. act. of the tumor cells is more constant and much higher than that of the serum. The spec. act. of the serum was about 4–7 per cent of that of the cells, calculated per unit of wet substance. It follows that the cells accumulate the labeled amino acid rapidly after its injection and against a strong concentration gradient, in agreement with the findings of Christensen and associates (3, 4).

Studying the spec. act. of the various biochemical fractions as a function of time it appears that the activity of the Ba-soluble fraction decreases at an approximately linear rate. About 30 per cent of the total cell activity found at 35 min. is present as Ba-soluble fraction, at 100 min. this figure is about 15 per cent. Concomitantly with this decrease all other fractions that have been analyzed were found to incorporate activity at an increasing rate. With DNA a slight "lag" could be observed in the incorporation rate 35 min. after irradiation; on the other hand ATP-ADP obviously incorporated ^{14}C at a very high rate during the time interval 0–35 min., whereas the incorporation in the period 60–135 appears to proceed at a slower rate. Apart from these exceptions incorporation proceeds at a nearly linear rate.

To give a general survey of the distribution of activity after irradiation as a function of time and in comparison to the unirradiated controls, the results were plotted in the following way (Fig. 2).

For each biochemical fraction a "relative spec. act." was computed by dividing the spec. act. of the fraction by the spec. act. of the whole cells. This was done separately for each series of experiments. The percentage change in the relative spec. act. of the X-rayed samples as compared to the corresponding controls was calculated and the average values for all analyses performed were plotted against time. The procedure seems to be justified as there was no appreciable difference between the spec. act. of the whole cells in the corresponding irradiated and control samples (cf. Table I).

Fig. 2 gives a general survey of the distribution of the total intracellular activity among the various fractions, provided that there has been no change in the chemical composition of the cells after irradiation. As shown above (Fig. 1) this assumption does not hold for the ATP-ADP fraction. The *total activity* present in ATP-ADP of the X-rayed cells at 35–60 minutes after treatment is not significantly lower than the control value. All other fractions studied, with the exception of the Ba-soluble one, show a depression of the relative spec. act. 35 minutes after irradiation. The slight surplus activity in the Ba-soluble fraction does not seem to counterbalance the strong deficit in the four other fractions, taking into account that only some 30 per cent of the total activity is present in that fraction. The explanation for this discrepancy is obscure.

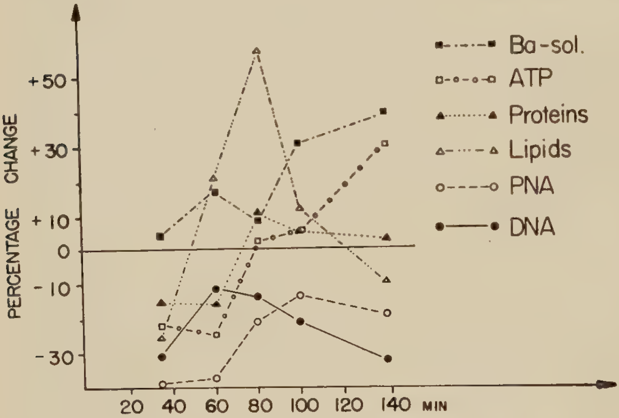

Fig. 2. Percentage change in the spec. act. of the fractions in irradiated cells compared with controls.

A different behavior after irradiation of the nucleic acids as compared to the Ba-soluble fraction (including ATP-ADP) is apparent when comparing the slopes of the curves in Fig. 2. The slight depression of proteins returns rapidly to normal conditions while the lipid curve indicates rapidly changing disturbances shortly after irradiation.

Correlations between ^{14}C- incorporation and the physiological age of the cells.—Variations in the physiological age of ascites tumor cells are correlated with changes in their total number and mass. As the amount of labeled glycine injected was kept constant in the present study, the same activity was distributed among an increasing number of cells with increasing age of the tumor. Hence follows the inverse correlation between the total weight of the tumor cells and their spec. act. It could be expected that the spec. act. of the various chemical fractions, too, will be influenced by the varying pool of activity. As the following evidence will show, this is in fact the case. Different fractions show a quantitatively different behavior, however.

TABLE IV

Ratios between the specific activities of cells with different physiological age.

1 Ratio of spec. act. in group:	2 Wet cells	3 Protein	4 DNA	5 PNA	6 ATP-ADP	7 Ba-soluble
			Controls			
$^1/_3$	4.00	6.79	11.1	4.03	3.79	2.37
$^1/_2$	2.14	3.20	4.69	2.63	2.18	1.99
$^2/_3$	1.86	2.13	2.37	1.53	1.73	1.19
			X-rayed			
$^1/_3$	3.68	5.70	9.43	4.14	3.66	3.15
$^1/_2$	1.78	2.52	4.40	2.41	1.95	2.14
$^2/_3$	2.06	2.26	2.14	1.72	1.87	1.47

The ratios between the spec. act. of the corresponding fractions from groups 1–3 with different physiological age are shown in Table 4. It appears that whole cells differ in their spec. act. by a factor of 4 at maximum (comparing groups 1 and 3), while the corresponding figures for protein and DNA are considerably higher. PNA and ATP-ADP, on the other hand, incorporate activity in proportion to the total pool of the isotope in the cell. The behavior of the Ba-soluble fraction shows a trend opposite to protein and DNA, as the ratios are appreciably lower than those for total cell activity in most cases. In the X-rayed samples the ratios are slightly higher than in the controls for the Ba-soluble fraction while the opposite trend is shown by proteins and DNA. This suggests a slightly stronger X-ray effect on the latter fractions in young as compared to older cells. For DNA, this was already indicated above (Table III).

The ratio between the spec. act. of PNA and DNA is also different for the three groups studied. The average ratio for groups 1, 2 and 3 was 1.51, 2.89, and 3.97 respectively. The difference between any pair of these values is statistically significant at the 0.1 per cent level.

The differences in the distribution of the isotope in cells with different physiological age suggest differences in metabolic rates, possibly related to the differences in growth rate. When comparing growth rate and isotope incorporation proteins, PNA and DNA are the compounds that should be considered in the first place among those studied, as they are the most important contributors to the organic mass of the cell. Previous studies (14) showed that the Ehrlich ascites tumor cell contains 230 $\mu\mu$g protein, 36.4 $\mu\mu$g PNA and 16.4 $\mu\mu$g DNA on an average. Proteins thus make up 81 per cent of the total amount of these three compounds. When comparing growth rate and isotope incorporation the behaviour of the protein fraction is the determining factor.

An "average ratio of spec. act.", taking into account the varying amounts of all the three compounds and using the ratios between youngest and oldest unirradiated cells in Table IV (Group 1 to 3), would be equal to

$$\frac{(230 \times 6.79) + (36.4 \times 4.03) + (16.4 \times 11.1)}{282.8} = 6.67.$$

The corresponding figures for the ratio between group 1 and 2 and between group 2 and 3, respectively, would then be 3.21 and 2.08. As seen from Table IV, col. 3, these figures agree closely with those for the proteins alone as could be expected. These figures for the proteins can be compared to the ratios of the total cellular spec. act. (col. 2, Table IV), which latter represents the average of the spec. act. of all compounds that have incorporated ^{14}C. When such a comparison is made it appears that there is a surplus of activity in the protein fraction.

A quantitative measure of this surplus is obtained by dividing the average ratio of the spec. act. of that fraction by the corresponding ratio of total cellular activity. The figures obtained are given in Table V, and they are compared to the ratios of relative growth rate. It appears that there is a rather close correspondence between the values obtained. This would support the conclusion that the incorporation rate of ^{14}C into the protein fraction of cells of different physiological age is closely related to the growth rate of the cells.

Preliminary experiments with cells of similar specific activity.—In experiments under way the amount of isotope injected was matched to the total number of tumor cells available in the animal, and thus samples with different physiological age but with almost identical cellular spec. act. could be obtained. Some preliminary results indicate that at least the proteins of physiologically younger cells incorporate more activity under these conditions. A reasonably good correlation between the relative growth rate and the ^{14}C-incorporation is also obtained (Table VI).

^{14}C-permeability of X-rayed and control cells.—Permeability changes, occurring shortly after the irradiation, may possibly have contributed to

TABLE V

Comparison between the surplus activity of proteins in young as compared to old cell with the ratio of their respective growth rates.

Groups	Surplus activity of protein fraction (Ratio of spec. act. of the fraction divided by ratio of total cellular activity)	Ratio of relative growth rates $\left(\frac{dN}{dt}\cdot\frac{100}{N}\right)$
$^1/_3$	$\frac{6.79}{4.00} = 1.70$	$\frac{2.12}{1.22} = 1.74$
$^1/_2$	$\frac{3.20}{2.14} = 1.50$	$\frac{2.12}{1.50} = 1.42$
$^2/_3$	$\frac{2.13}{1.86} = 1.15$	$\frac{1.50}{1.22} = 1.23$

TABLE VI

Spec. act. of proteins from cells of varying physiological age but with constant cell activity. Comparison between incorporation of activity into protein fraction and growth rate of the cells.

Group	Counts/min/mg of:		Ratio of protein act.	Ratio of rel. growth rates $\left(\frac{dN}{dt}\cdot\frac{100}{N}\right)$
	Wet cells	Proteins		
1	27.0	74	$\frac{74}{44} = 1.68$	$\frac{2.52}{1.40} = 1.80$
2	28.6	67	$\frac{67}{44} = 1.52$	$\frac{2.00}{1.40} = 1.47$
3	28.8	44	$\frac{74}{67} = 1.10$	$\frac{2.52}{2.06} = 1.22$

TABLE VII

Spec. act. of tumor cells and ascites serum 5 minutes after the injection of ^{14}C-glycine.

Group	Total activity in:		Spec. act. (counts/min/g) in:	
	Cells	Serum	Cells	Serum
Controls. . .	2330	1410	1100	500
X-rayed . . .	2250	1320	1010	570

the low spec. act. values at 35 min. (Fig. 2). Animals were therefore sacrificed exactly 5 min. after injection of a tracer dose of glycine. The ascites was collected and cells and serum were separated in an ice bath by the aid of a rapid microcentrifuge in further 2 min. Mice with a fairly large amount of ascites per animal were used in order to reduce the experimental errors. The results calculated per mouse (mean of 8 mice in each group) do not give support to the permeability hypothesis (Table VII).

DISCUSSION

The present investigation has demonstrated that X-irradiation with a dose of 1250 r produces certain changes in the pattern of incorporation of glycine-2-^{14}C into the proteins, lipids, nucleic acids, ATP-ADP and a complex carbohydrate fraction ("Ba-free residue") of Ehrlich ascites tumor cells.

The relative stability of the protein fraction after irradiation is in agreement with earlier investigations. Using ^{14}C-labeled acetate, Hevesy found a slight increase of ^{14}C-incorporation into the proteins of various organs eight hours after irradiation (9). This was probably due to the fact that the metabolism of ^{14}C-acetate to $^{14}CO_2$ is decreased in X-rayed animals shortly after irradiation, producing a consecutive net increase of the amount of ^{14}C-acetate available for protein synthesis a few hours later. Holmes (12) and Appleyard (2) found no difference in the incorporation of ^{35}S-sulphide and ^{15}N-labeled glycine, respectively, into the proteins of irradiated and untreated Jensen rat sarcoma at short time intervals (60–90 min.) after irradiation. The depressive effect found in the present work is rapidly reversed and in the interval 60–150 min. the incorporation rate is similar to the controls.

Irradiation produces a pronounced alteration in the incorporation of labeled glycine into the lipid fraction, as demonstrated by the present experiments. Previously, Hevesy *et. al* (10) found a decreased incorporation of ^{14}C-acetate into liver lipids 5–30 min. after irradiation, but increased values after 90 minutes. Using ^{32}P, the same author (7) described a markedly decreased phosphatide turnover in liver and sarcoma tissue 2 hours after irradiation. All these findings are compatible with our lipid curve (Fig. 2) which shows that the nature of the changes found will primarily depend on the time after irradiation at which the analysis is made.

The depressive effect of irradiation on the incorporation of labeled precursors into DNA is well established from many investigations and is confirmed by the present work (cf. 6, 8, 11). In as yet unpublished studies concerning biochemical changes during the postirradiative amitotic period and the early stage of reappearing mitotic activity in the Ehrlich ascites tumor, we have found that the depression of glycine incorporation into DNA at 100 minutes, as described in the present paper, is maintained at about the same level for nearly 30 hours after irradiation. Mitotic figures disappear completely within 2 hours after irradiation, and do not reappear until about 16 hours later (14). As there is no division and no detectable cell death during this period, the cell population remains constant. The amount of DNA synthesized during this period is only 32.5 per cent of what is produced by a comparable unirradiated cell population as shown in the previous work (14). Incorporation of labeled glycine into DNA proceeds, on the other hand, at a rate of about 70–75 per cent on an average. There is thus a surplus incorporation of glycine into DNA which is not accounted for by a net increase in the number of DNA molecules. This is analogous to the findings of Euler and Hevesy (6).

The amount of PNA synthesized during the amitotic period was similar to the increase in unirradiated control population (14). In apparent contradiction to this, the incorporation of glycine into PNA was found in the present work to decrease considerably during the first two hours after irradiation. Experiments under way indicate, however, that the incorporation of activity into PNA gradually approaches normal values during the subsequent amitotic period although only after an appreciable "lag", and occasionally showing cyclic changes.

Available evidence indicates that cellular PNA is metabolically heterogeneous. Thus, Jeener and Szafarz (13) found that ^{32}P-incorporation into PNA proceeds at a different rate in three different cytoplasmic fractions. Marshak (19) showed that nuclear PNA incorporated ^{32}P at a much more rapid rate than cytoplasmic PNA. The effect of X-rays on different PNA fractions may also differ, as indicated by recent investigations (20).

Earlier work (1, 11) has shown, that the incorporation of ^{32}P into the total PNA fraction of the Jensen rat sarcoma is much less affected by irradiation than the corresponding DNA process, even when the first hour after irradiation is considered. The different result obtained in the present investigation could be due to differences in the precursor and tumor used.

Payne *et al.* (21) and Tyner *et al.* (24) have summarized recent investigations concerning the relative incorporation of ^{32}P and ^{14}C from various precursors into the two types of nucleic acids. The available evidence indicates that the rate of incorporation of different isotopes into DNA is independent of the nature of the precursor, while incorporation into PNA shows considerable variations. The ratio between the spec. act. of PNA and DNA was found to be rather high when ^{32}P was used as a tracer while experiments with ^{14}C-glycine yielded lower values.

In our experiments low values were obtained in general (p. 489). As the spec. act. of PNA was independent of cell age and/or correlated variations of isotope concentration while DNA was not, the PNA/DNA ratio varied correspondingly between the different experimental groups. Irradiation did not change the ratio for a given experimental group, however, as seen from cols. 5–6, Table I (cp. also Table III). It may be speculated that in cases where the incorporation rate into the two types of nucleic acids is similar, irradiation influences them in a quantitatively similar way.

If relative incorporation rate is plotted as a function of time for the various fractions investigated in the present work (cf. Fig. 2), it appears that irradiation is followed by a depression of activity levels for the proteins, nucleic acids and lipids which is obvious 35 minutes after treatment and lasts until 60–80 minutes after irradiation when the tendency is reversed in the case of nucleic acids and lipids. Metabolic changes or radiation effects on cellular permeability and isotope distribution are alternative explanations for the phenomena observed.

The intraperitoneally injected isotope was available for the freely suspended ascites tumor cells primarily and immediately and had to pass through only one cell membrane. This, together with the fact that the total cellular activity agreed closely in irradiated and control samples in samples analyzed already 5 min. after the injection of the isotope (Table VII) as well as in the main experiments (Table I) makes it improbable that X-ray effects on permeability and isotope distribution could have influenced the results to any major degree. It is more probable that the findings are due to metabolic disturbances. Assuming that this is the case it may be suggested that irradiation has retarded the formation of precursors and thereby produced a certain "lag". A rapid reversal of the changes is indicated by the rising trend of the curves between 35 and 80 minutes after irradiation. In most cases the rise is only temporary, however, and is followed by subsequent decrease.

The behavior of the ATP-ADP fraction—which consists predominantly of ATP—is of particular interest. It may be suggested that irradiation inhibits the consumption of ATP as an early effect, thereby leading to an increased cellular concentration of this substance. Assuming that the accumulation of ATP starts very soon after irradiation, its low spec. act. at 35 minutes can be explained as an "isotope dilution". This interpretation implies that the cell will be deprived of an essential energy-delivering system for a period of time with a consecutive delay of synthetic reactions, such as e.g. the formation of precursors of proteins and nucleic acids.

The initial inhibition seems to be followed by an increased consumption of ATP, as suggested by the shape of the curve denoting the cellular ATP-concentration (Fig. 1) during the period between 35 and 100 minutes after irradiation. The simultaneous rise in the spec. act. of the various compounds of high molecular weight studied is in agreement with the interpretation presented above and suggests that X-ray effects on ATP may determine the course of the initial biochemical changes after treatment.

Using ^{32}P it has been shown previously (23) that the spec. act. of ATP in mouse liver is increased as early as 5 minutes after irradiation. A still more pronounced increase was found after 24 hours. No determination of ATP-concentration were performed. In the present material spec. act. of ATP was found to be decreased during the early period after irradiation while unpublished evidence shows that several hours after treatment there is an increased incorporation of ^{14}C into the ATP fraction of irradiated cells as compared to the controls. Experiments are under way to decide whether the discrepancy during the early postirradiative period is due to differences in the biological material (liver versus ascites tumor) or to the fact that X-rays effect the incorporation of ^{32}P and ^{14}C, respectively, into ATP in a different way.

The differences in the cellular distribution of ^{14}C as correlated to the physiological age of the cells might be due to metabolic differences between young and old cells and/or to the systematic variation of cellular spec. act. in samples with different age. In some preliminary experiments the amount of isotope injected was matched to the total number of tumor cells available in the animal and thus samples with different physiological age but identical (within ± 5 per cent) cellular spec. act. could be obtained. Under these conditions young cells incorporated ^{14}C at a higher rate into the protein fraction than old cells, similarly to what was the case under the conditions of the present experiments. It is therefore reasonable to assume that metabolic differences related to the physiological age of the cells mainly caused the differences in ^{14}C incorporation as described in the present paper.

As the concentration of activity in the ascites serum is low compared with that of the cells (p. 487), the anabolism of the proteins and nucleic acids proceeds mainly by utilising the isotope that is available within the cells. Calculations based on this assumption gave a rather good agreement between the ratios of the relative growth rates of cells with different physiological age and the ratios of ^{14}C-incorporation into the protein fraction.

SUMMARY

1. The Ehrlich mouse ascites tumor was irradiated *in vivo* with 1250 r X-rays. The incorporation of ^{14}C-glycine into various cellular components was studied during the period between 35 and 150 minutes after irradiation and was compared to unirradiated controls. Determinations were made of the total activity of tumor cells and ascites serum per unit weight, and of the spec. act. of ATP-ADP, PNA, DNA, proteins, total lipids and the Ba-soluble and Ba-insoluble fractions of trichloroacetic acid (TCA)-extracts.

2. Irradiation produced pronounced changes in the incorporation rate of ^{14}C into ATP-ADP, the nucleic acids, proteins, lipids, and a complex carbohydrate fraction. These changes were dependent upon the time that elapsed between the injection of the isotope (which took place immediately after irradiation) and the killing of the animals. There was a strong depression of activity levels of the nucleic acids, proteins, and lipids 35 minutes after irradiation. This was followed by a temporary increase between 35 and 80 minutes in case of the nucleic acids and lipids.

3. Irradiation did not change the permeability of the tumor cells to the isotope. The low activities found in various compounds 35 minutes after irradiation were thus not due to alteration of cellular permeability.

4. The cellular concentration of the ATP-ADP fraction, consisting predominantly of ATP, was increased 35 minutes after irradiation by about 25 per cent. It is suggested that the consumption of ATP is inhibited soon after irradiation. This might lead to a delay of precursor formation for nucleic acid, protein and lipid synthesis, resulting in their decreased spec. act. During the interval between 35 and 100 minutes after irradiation, an increased consumption of ATP occurs concomitantly with a rise of spec. act. in the highmolecular compounds. The low spec. act. of ATP at 35 minutes can be interpreted as "isotope dilution", while the subsequent increase of activity during the time interval 35–100 minutes indicates that ATP-synthesis is not impaired. It appears that X-ray effects on ATP-utilization may possibly determine initial biochemical changes after irradiation.

5. Irradiation was followed by a decreased ^{14}C-incorporation into both PNA and DNA to about 75 per cent of the control value. The similar X-ray effect on both nucleic acids might be related to the fact that the incorporation rate of labeled glycine into both compounds is quantitatively similar in the control material. Since total DNA-synthesis in irradiated cells is only 33 per cent of the controls, there is a surplus incorporation of ^{14}C into DNA which is not accounted for by a net increase of DNA-molecules.

6. There was a good agreement between the relative growth rate of tumor cells with varying physiological age and the rate of ^{14}C-incorporation into the protein fraction.

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THE ABSORPTION OF PROFLAVINE BY GRAM-POSITIVE AND GRAM-NEGATIVE ORGANISMS

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In a previous study (11) the absorption of proflavine (2:8-diaminoacridine sulphate) by a Gram-negative organism, *Bact. lactis aerogenes* (*Aerobacter aerogenes*), was measured under various conditions. Not unexpectedly, certain features of growth in the presence of this drug and of the process of acquiring resistance to it were found to be more simply related to the amount of proflavine actually absorbed by the cells than to its concentration in the growth medium. In particular, the absorption of proflavine per cell was found to change very little over a pH range (6.16–6.96) in which increase of hydrogen ion concentration markedly decreased its effect on the duration of the lag phase (12). This suggested that the centres where absorption of proflavine cations can cause prolongation of lag are only a small fraction of the total which are capable of absorbing the drug and differ from the bulk in their greater sensitivity to changes in pH in the range mentioned.

In general Gram-positive organisms are known to absorb basic dyes more readily than Gram-negative ones. This is apparent in the lower "isoelectric zones" of Gram-positive organisms (*c.* pH 2) compared with those of Gram-negative organisms (*c.* pH 5–6) (14). The "isoelectric zone" is the range of pH within which acidic and basic dyes are retained to approximately the same extent. At pH values above this zone the absorption of a basic dye rises to a constant maximum value. These observations suggest that Gram-positive organisms should be the more sensitive to bacteriostatic cations and this has been observed with the triphenylmethane dyes (15), although the gradation is continuous in a series of organisms and there is no clear cut division into two groups of widely differing sensitivity. The usual initial pH of growth in many bacterial cultures (*c.* pH 7) is above the isoelectric zones of both Gram-positive and Gram-negative organisms. Under these conditions both Gram-positive and Gram-negative cells should absorb maximum amounts of bacteriostatic cations, and it is not clear what difference in sensitivity is to be expected. Indeed, a study of the average inhibitory dilutions of six highly ionized acridines, including proflavine, towards a series of Gram-positive and Gram-negative organisms has shown only a slightly greater sensitivity in the former (1). A better correlation was found between the exacting nature of an organism's nutritional requirements and its sensitivity to the acridines.

It is clearly desirable, in this context, to have more precise information than is available from staining experiments about the actual uptake of acridines by organisms differing in their sensitivity to the compounds and in their Gram staining behaviour. It should then be easier to find any relationships between these various properties of bacterial cells. In continuation of previous work, the absorption of proflavine by living saline suspensions at pH 7 of two Gram-positive and two Gram-negative organisms has therefore been measured. Proflavine has the advantage that it is absorbed by both living and dead cells (2), and hence loss of viability in cell suspensions kept in the presence of this compound should not profoundly affect the comparisons. Since cells of different species vary considerably in size and shape, the absorption per mg dry weight of the various organisms was measured. Parallel studies were also made of the dry weight per cell, and of the sizes of the organisms. The absorption of the Gram-negative "cytoskeleton" obtained by cholate extraction (6) of one of the Gram positive organisms (*Cl. Welchii*) was also determined.

EXPERIMENTAL

Organisms and growth conditions

Gram-positive. I. *Cl. welchii* (*Cl. perfringens*). This was an avirulent strain which was grown under conditions of reduced oxygen tension at 37° C on a liquid medium consisting of: glucose, 10 g/l; sodium chloride, 5 g/l; "Lemco" broth, 5 g/l; Difco "Casamino" acids, 30 g/l; peptone, 1–10 g/l (pH 7.0).

II. *Strep. faecalis.* The streptococci were grown aerobically at 37° C on a liquid medium consisting of: glucose, 2 g/l; sodium chloride, 5 g/l; "Lemco" broth, 5 g/l; Difco "Casamino" acids, 10 g/l; peptone, 5 g/l.

Gram-negative. III, *Bact. lactis aerogenes* (*Aerobacter aerogenes*), maintained by serial transfer of the culture used in the previous absorption study (11), and IV, *Bact. coli* (*Esch. coli*), were obtained originally from the National Collection of Type Cultures. III and IV were grown aerobically at 37° C in a liquid medium consisting of: glucose, 50 g/l; ammonium sulphate, 5 g/l; magnesium sulphate, 1 g/l; potassium dihydrogen phosphate, 9 g/l, together with sufficient concentrated sodium hydroxide to bring the pH to 7.1.

The media constituents were as chemically defined as was found to be consistent with obtaining a stationary population sufficiently large for subsequent use. The rate of growth and the relationship between the age of a culture and the duration of its lag phase on subculturing into fresh media were determined for I, II, and III by the usual methods (5). This information, which was already available for IV, made it possible to control the period at which a given culture would reach the end of its logarithmic phase. It was often found convenient to use as a measure of growth the turbidity of the growing culture, as recorded on a "Spekker" absorptiometer (water as standard; neutral filter, H 509).

Determination of absorption of proflavine. When a 4 l culture of one of the organisms reached the end of its logarithmic phase, it was centrifuged at 25,000 r.p.m. (Sharples centrifuge) and the separated cells were washed with 1 l of saline (9 g/l) and then mixed with 30 ml of fresh saline to give a very thick suspension (A). The suspension A was made homogeneous and any adhering masses of cells were broken up by the passage of a rapid stream of air or by mechanical stirring, as in the case of I. From the measured turbidity of a suitably diluted sample of A an estimate of the dry weight of bacteria per ml was obtained from a calibration curve, previously constructed in separate experiments (for determination of bacterial dry weights, see below). A portion of A was then diluted so as to give a suspension (B) containing about 1500–2000 mg dry weight bacteria per litre. 5 ml portions of B were transferred to 6″ × 1″ test tubes containing 10 ml of phosphate buffer (pH 7.1, as used in the media for III and IV), x ml of a 1000 or 2000 mg/l solution of proflavine hemisulphate (British Drug Houses Ltd., London), and (10-x) ml of saline (9 g/l). The final bacterial concentration was thus 300–400 mg dry wt/l, which experiment showed to be the most suitable. Values of x were chosen to give final proflavine concentrations in a range 300 800 mg/l, which enabled the organisms to absorb to their maximum extent. The tubes were maintained at 37° C, the temperature of growth, gently aerated to keep the mixture stirred, and were shielded from daylight to minimise any proflavine decomposition. The absorption of proflavine increased gradually with no breaks, such as might have occurred on the death of the cells, and when equilibrium had been attained (3 hours for I and II, and 1 ½ to 2 hours for III and IV, as shown in separate experiments) the suspensions were centrifuged for 15 minutes at 6000 r.p.m. The concentration of unabsorbed proflavine in the clear supernatant was determined by means of the Spekker instrument and a previously measured calibration curve (blue filter 601; a 1 g/l potassium chromate solution was a convenient stable standard). From these determinations and the initial total concentrations, the amount of proflavine absorbed (in mg/l) could be calculated.

The dry weight of bacteria per ml of solution in the absorption tubes was calculated from the known dilution factors and from the bacterial concentration in A. The latter was determined directly by measuring out a given volume (usually 4 ml) of A into each of three small, dried, and weighed crystallising dishes and an equal volume of saline into two other similar weighed dishes. All five were then evaporated to constant weight at 85° C, at which temperature no charring occurred. After subtraction of the weight of sodium chloride present, the readings gave the dry weight of bacteria per ml of A and hence also per ml of the absorption suspension. The number of mg of proflavine absorbed by 1 mg dry weight of the organisms (A_w) could then be calculated.

Determination of the dry weight per cell. Thick suspensions of each organism were prepared and the dry weight concentrations determined as above. Samples of the same suspensions were suitably diluted and the number of cells per ml counted using a Thoma haemocytometer. With II, groups of two joined cocci were counted as one cell. These measurements then gave the dry weight per cell.

Cholate extracted Cl. welchii. Cells of I were harvested as previously described and were suspended in a 20 g/l solution of sodium cholate at 60° C until test samples showed that all the cells were Gram-negative (after about 44–48 hours). The cells were re-centrifuged, washed, and suspended in saline and their absorption of proflavine at 37° C was measured. It was not found possible to render II Gram-negative by these methods.

Bacteriostasis. The concentration of proflavine required to produce an infinite lag, i.e. to inhibit growth completely, was determined for each of the four organisms when growing under the conditions described.

Bacterial size. In order to obtain some direct measure of the relative surface areas and volumes of the organism, electron micrographs were taken and the mean values of the greatest and least dimensions of the cells were obtained.

RESULTS AND DISCUSSION

The main experimental results are given in Table I. The absorption, A_w, reached a maximum when the concentration of free proflavine was greater than 200 mg/l and it is these maximum values that are recorded in Table I. The absorption decreases in the order I > II, III > IV and the differences so represented are statistically significant (i.e., the actual differences in A_w are more than 10 × the probable errors in these differences). The graded decrease in absorption per cell, which is calculated from A_w and the weight per cell, can be chiefly ascribed to the decrease in this last quantity along the series I to IV. The Gram-positive organisms do not differ markedly from the Gram-negative organisms in absorptive power (A_w) towards the acridine and II and III actually have the same maximum value of A_w. *Cl. welchii* is one of the mostly intensely Gram-positive organisms, yet its proflavine absorption is only about 70 per cent higher than that of Gram-negative *B. coli.* Both classes of organisms readily absorb the acridine at pH 7 and both are completely inhibited by concentrations which are of the same order.

There appears, therefore, to be no basis for drawing any striking contrast between Gram-positive and Gram-negative organisms in their behaviour towards proflavine. It has usually been assumed that Gram-positive organisms absorb cationic dyes more readily than Gram-negative ones and there is good evidence for this at lower pH's (15). However, the values of A_w reported here show that near pH 7, the pH of most growth experiments, the differences in uptake of the proflavine cation are not very great. Moreover, even if a marked contrast in absorption of the cationic drug could have been demonstrated for the two classes of organism, it could not necessarily have been

TABLE I

Organism	I *Cl. welchii*	II *Str. faecalis*	III *Bact. lactis aerogenes*	IV *Bact. coli*	V Cholate extracted I
Gram staining	+	+	−	−	−
Maximum A_w (mg proflavine absorbed/mg dry cells)	0.24 ± 0.02* (33)	0.18 ± 0.02 (43)	0.18 ± 0.03 (40)	0.14 ± 0.02 (32)	0.13 ± 0.01 (17)
Maximum absorption (mg) proflavine per cell $\times 10^{10}$	7.1	5.0	1.7	1.1	
Inhibitory conc. of proflavine (mg/l)	*c.* 20	*c.* 20	55	50	
**Phosphorus content (mg P/mg freeze dried cells) $\times 10^2$	2.05	1.78	2.08	2.55	0.68

* Mean ± probable error of mean; no. of independent measurements below in parentheses.
** Ref. 10.

TABLE II

Organism	I	II	III	IV
Length (μ)	2.8	1.2	1.8	1.2
Breadth (μ)	0.9	0.6	1.0	0.5
Vol. (μ^3)/cell	2.3	0.30	1.1	0.20
Surface area (μ^2)/cell	11	2.3	5.5	1.9
Absorption of proflavine/unit vol. (mg/μ^3) $\times 10^{10}$	3.1	16	1.5	5.5
Absorption of proflavine/unit surface (mg/μ^2) $\times 10^{10}$	6.5	2.2	0.31	0.55

correlated directly with the bacteriostatic action of the drug, since the larger proportion of proflavine molecules appear to be absorbed at sites not metabolically active (11). This suggestion is in agreement with the identity of the absorptive powers of II and III in contrast with the distinctly greater sensitivity of II to the drug.

Cholate extraction of *Cl. welchii* renders the organism Gram-negative and reduces but does not eliminate its absorption of proflavine; this indicates that it is not the Gram-staining sites only which are involved in proflavine absorption. There is evidence (10) that, under the conditions employed, sodium cholate extracts nearly all of the nucleic acids of *Cl. welchii* and leaves a residue which fails to give the Dische test for deoxyribonucleic acid (9). Both nucleic acid and non-nucleic acid components must absorb proflavine and this is in accordance with the lack of correlation between the A_w values and the phosphorus contents (Table I) of the same organisms (10). This is of interest in view of the possible connection between the bacteriostatic action of proflavine and the nucleic acid components of the cell (8). Proflavine interacts very strongly with deoxyribonucleic acid in solution (13) and other acridines react similarly with ribonucleic acids (7). It has also been shown that proflavine interferes with the synthesis of deoxyribonucleic acid in *Bact. lactis aerogenes* (3) and a similar deduction has been made from the proflavine inhibition of bacteriophage formation in *B. coli* (4).

The preceding discussion suggests that no single feature of the bacterial cell can be demonstrated to be all-important in determining proflavine uptake. This is further emphasised by some calculations based on cell dimensions as determined by electron microscopy (Table II). Dimensions so obtained are subject to considerable uncertainty because of the unknown effect of dehydration on actual and relative cell sizes. However, the dimensions of Table II are similar to those obtained by microscopic techniques and some further tentative calculations seemed justified. Assuming that the cells approximated to short rods with hemispherical ends (a reasonably good model for I, III, and IV but only a crude approximation for II), the volumes and surface areas per cell could be calculated; hence, from a knowledge of the absorption per cell (Table I), the absorption of proflavine per unit of cell surface and cell volume was obtained. The values (Table II) show no apparent regularities amongst themselves or in comparison with the other observations.

It must be concluded that no single property of an organism, not even its Gram staining behaviour, can be regarded as the determining factor in the absorption of proflavine, which appears to be only partly taken up on the nucleic acid components of the cells.

SUMMARY

The absorption of proflavine by saline suspensions of two Gram-positive and two Gram-negative organisms has been measured at pH 7 and 37° C. The absorption per unit dry weight of the organisms decreases in the order

(*Cl. welchii*) > (*Str. faecalis, Bact. lactis aerogenes*) > (*Bact. coli*), although the differences are not striking. There appears to be no correlation of absorptive power with any single property of the cell such as Gram-staining behaviour, inhibitory concentration of proflavine, phosphorus content, cell volume or cell surface.

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THE MECHANISM OF CELL DIVISION: EXPERIMENTS WITH ETHER ON THE SEA URCHIN EGG

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A RECENT paper by Swann (13) described experiments on the delays in the cleavage of sea urchin eggs (*Psammechinus miliaris*) induced by inhibition of the cytochrome system with carbon monoxide. Two important points emerge from this work: firstly that with inhibitions beginning at any time up to about 35 minutes after fertilisation, the delay in cleavage is equal to the period of inhibition; secondly that with inhibitions beginning after about 35 minutes, the first cleavage is unaffected, while the delay in the *second* cleavage is equal to the period of inhibition.

These facts can best be explained by supposing that the cell contains some sort of reservoir which is steadily being filled by normal aerobic respiration. When the reservoir is full, at about 35 minutes after fertilisation, the contents siphon out and start off the processes of mitosis and cleavage, which then continue, regardless of whether the energy supply is functioning or not. In the normal course of events, however, the reservoir continues to fill while the first mitosis and cleavage run through; when it is full again, after a further 35 minutes, the siphoning out starts off the next mitosis, and so on. On this basis, successive mitoses and cleavages should be separated by the length of time taken for the reservoir to fill, namely about 35 minutes, and with the eggs of *Psammechinus miliaris* at the temperatures in question, this is the case. The hypothesis also accounts for the fact that oxygen consumption fluctuates only slightly, or not at all during the division cycle (11, 14), since the energy requirement is continuous.

The idea of a reservoir mechanism raises a number of problems. In particular it would be desirable to get confirmation of the idea by other means, and to know more about the nature of the reservoir. Is it, for instance, a store of energy, or of some non-energy carrying compound? Narcotics seemed to offer a possible means of approach to these problems. They are well known as reversible inhibitors of cell division, so that they can be used for quantitative work on the lines of the carbon monoxide experiments. They are also known to cause a solation of the mitotic apparatus, and it seemed possible that if the store were not a simple chemical affair, it might be associated with certain of the oriented protoplasmic structures in the cell. It may be significant, for instance, that at the critical time of 35 minutes or so, the sperm aster (or in later mitoses the interphase aster) quite suddenly disappears. Of the many possible narcotics, ether was chosen as being likely to give rapid and complete reversibility of effect.

MATERIAL AND METHODS

The eggs of the sea urchin *Psammechinus miliaris* were used throughout the experiments. They were inseminated at sperm densities high enough to ensure that fertilisation was complete within one minute (10), and washed twice. They were then treated with 1 and 2 per cent solutions of ether (diethyl ether) in sea water, at various points in the mitotic cycle, and for varying lengths of time. To get rid of the ether, the supernatant fluid was drawn off and the eggs were then resuspended in sea water; this was repeated three times over, and took about four minutes.

Shortly before division a small number of eggs were placed in a drop of water on a microscope slide and photographed with a 16 mm time lapse camera at intervals of one minute. The magnification was arranged to give a field of about 50 eggs. The film was analysed with a ciné-viewer, and the time at which each egg began its first, and in a few cases its second cleavage was recorded. The criterion used for the start of cleavage was the appearance of a distinctive furrow or 'V'. Unlike the preliminary lengthening of the cell, this stage can be distinguished even when the axis of the cell coincides with the axis of vision (13). From these records the average time of cleavage was determined for each batch of eggs.

In addition, a number of observations were made with a polarising microscope, to determine whether or not mitotic figures were present at particular stages in the experiments (12).

RESULTS

Altogether forty-four experiments were done (excluding controls) using the eggs of four different females; each experiment involved about fifty eggs. The temperature varied between 17.8° C and 19.2° C. Tables I and II give the results in a condensed form; Text-figs. 1–4 show the delay in time of cleavage against the duration of inhibition, for different times of onset of inhibition.

The average percentage of cleavage in the controls was 94.5, and it will be seen from column 5 of Tables I and II, that the percentage of eggs cleaving or starting to cleave in the different experiments, was for the most part of this same order. In a few cases, however, the figure was much lower. There seems to be no very clear correlation between the percentage of eggs cleaving, and the time of starting or the duration of inhibition, but in a general way it is evident that the figures were lower when the inhibition started late in the mitotic cycle or went on for a long time.

TABLE I

One per cent ether experiments.

1 Start of inhibition, minutes after fertilisation	2. Duration of inhibition, minutes	3. Delay of cleavage, minutes	4. Time from end of inhibition to start of cleavage, minutes	5. Percentage of eggs cleaving or starting to cleave	6. Remarks on nature of cleavage
5	15	2.4	40.2	98	Normal
5	30	2.9	25.7	98	Rather multiple
5	45	7.9	15.2	98	Untidy
5	60	23.3	15.6	67	Untidy, many fail to complete
20	15	–1.8	21.0	96	Normal
20	30	7.4	15.2	83	Multiple, many fail to complete
20	45	25*	17*	Low	Many fail to complete
20	60	38.8	16.6	91	Multiple
30	15	4.9	17.2	98	Normal
30	30	19.2	16.5	53	Untidy, many fail to complete
30	60	46.6	13.9	96	Multiple
45	15	22.5	20.3	52	Nearly all fail to complete
45	30	35.9	18.7	86	Multiple
45	60	61.8	14.6	18	Nearly all fail to complete

Average time of cleavage in controls 57.6 minutes.

* Visual estimation.

The action of ether is evidently not always completely reversible. From column 6 in both tables it will be seen that the type of cleavage varied, from normal to somewhat abnormal. In the abnormal cases the cleavage patterns were irregular and untidy, and varying proportions of eggs failed to complete their divisions. Here again there is no very obvious correlation with the start and duration of inhibition, but the later and longer inhibitions were the most liable to give rise to abnormal cleavages. In a number of cases, particularly with late inhibitions, the cleavages were multiple. This is presumably because the centrosomes had divided in spite of the inhibition and so given rise to multiple asters.

In three cases the delay in cleavage had to be estimated visually because of cuts in the power supply. With this hazard of Marine Stations in mind, visual estimates were in fact made for every experiment, and they proved to be accurate to within a minute or two. (Mean error + 0.46 minutes, standard deviation ± 1.72 minutes.)

TABLE II

Two per cent ether experiments.

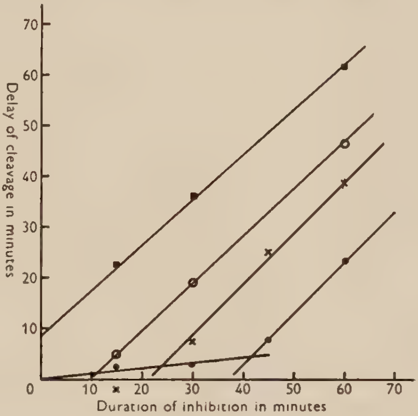
1. Start of inhibition, minutes after fertilisation	2. Duration of inhibition, minutes	3. Delay of cleavage, minutes	4. Time from end of inhibition to start of cleavage, minutes	5. Percentage of eggs cleaving or starting to cleave	6. Remarks on nature of cleavage
5	10	10.4	52.7	92	Normal
5	15	13.4	56.2	96	Normal
5	15	9.4	52.2	94	Normal
5	30	19.1	41.9	90	Normal
5	30	22.3	44.6	98	Normal
5	30	17.0	44.8	97	Normal
5	50	31.5	33.8	80	Normal
5	60	33.9	26.7	85	Normal
5	70	44.0	26.3	96	Normal
20	10	7.6	34.9	97	Normal
20	15	9.1	31.9	98	Normal
20	20	10.5	27.8	98	Normal
20	30	18.3	25.6	86	Normal
20	30	14.0	21.8	93	Normal
20	40	24.4	21.7	71	Many fail to complete
20	50	34.5	21.8	57	Many fail to complete
20	60	39.1	16.4	97	Untidy
20	60	43.2	21.0	36	Many fail to complete
20	80	64.6	21.9	60	Untidy
30	5	10.3	32.6	94	Untidy, multiple
30	15	12.1	29.9	100	Normal
30	20	16.9	24.2	100	Normal
30	30	24.9	27.7	98	Multiple
30	45	36.0	18.3	92	Untidy, multiple
30	60	50*	23*	High	Multiple
45	5	12.7	20.0	74	Many fail to complete
45	15	20.4	23.2	93	Normal
45	30	37.9	25.7	94	Multiple
45	60	61*	19*	High	Multiple
45	60	64.1	16.9	73	Untidy, multiple

Average time of cleavage in controls 57.6 minutes.

* Visual estimation.

A few experiments were also carried out on the effect of inhibition on *second* cleavage, on the action of concentrations of ether below 1 and above 2 per cent, and on the action of urethane.

The interpretation of the results is not altogether easy. The best starting point is perhaps the 1 per cent ether experiments, with inhibitions beginning at 5, 20 and 30 minutes (Text-fig. 1). In each case it is evident that a line can can be drawn through the higher points, making an angle of about 45° with the ordinate and abscissa. Each line, however, intercepts the abscissa (indicating nil delay) at a different point: about 35, 20 and 10 minutes, for inhibitions beginning at 5, 20 and 30 minutes respectively. The sum of the intercept and the starting time of inhibition comes therefore to about 40 minutes in each case. It is further evident that the delays caused by the shorter inhibition periods are only slight, and the lines to be drawn through these points are nearly horizontal. In short, the delays produced by inhibitions starting at 5, 20 and 30 minutes are more or less nil up to 40 minutes after fertilisation; thereafter they are more or less equal to the duration of the inhibition over and above 40 minutes.



Text-fig. 1. Duration of inhibition and delay of cleavage for 1 per cent ether. Inhibitions starting at:

5 minutes after fertilisation — solid circles
20 ,, ,, ,, — crosses
30 ,, ,, ,, — open circles
45 ,, ,, ,, — squares.

The emergence of a critical point at 40 minutes is interesting. The experiments were carried out between temperatures of 17.8 and 19.2° C, and gave an average time to first cleavage of 57.6 minutes in the controls. The previous carbon monoxide experiments were carried out at slightly higher temperatures, and gave somewhat shorter times to first cleavage. The critical point of about 40 minutes in the ether experiments must therefore correspond closely to the critical point of about 35 minutes in the CO experiments.[1]

The fact that inhibition occurring before 40 minutes produces little or no delay in the time of cleavage must of course mean that 1 per cent ether is without effect on the aerobic mechanisms concerned with filling the reservoir. It implies too that the oriented protoplasmic structure of the sperm aster is not an essential feature of the reservoir, for polarised light observations show that it is solated by ether at this concentration.

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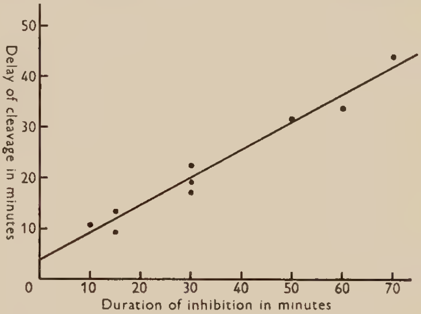
[1] Forty minutes at these temperatures is about the point at which the sperm aster and streak disappear, to be followed quite soon by the appearance of the asters and spindle. This time is perhaps to be considered as marking the end of interphase and the beginning of prophase, though some authors would regard it as being well into prophase.

At about 40 minutes however, it is evident that the egg moves into a new phase, in which, to judge from the previous CO experiments, the aerobic mechanism for filling the reservoir is no longer necessary for the completion of cleavage. At the temperatures in question, about 17 ½ minutes normally elapse between the start of this phase and the start of cleavage. It will be seen from column 4 of Table I that where inhibition extends up to or past the critical point a similar length of time elapses between the end of inhibition and the start of cleavage. The average for 8 experiments with inhibitions starting at 5, 20 and 30 minutes, is actually 15.9 minutes.

The action of 1 per cent ether, if inhibition is carried beyond 40 minutes, is therefore to hold the egg at or near the 40 minute stage. Whether or not the reservoir siphons out under these circumstances, its contents are not depleted, for mitosis continues as soon as the inhibition is lifted. On the other hand the contents do not apparently get built up further, or if they do, they are unable to force mitosis through any faster. Presumably the cytoplasmic and chromosomal aspects of mitosis are going ahead at the maximum possible rate in the normal course of events.

It is evident therefore that ether at a concentration of 1 per cent is preventing the building of the mitotic figure, *although the necessary preparations for it are complete.* There are a number of ways in which this might come about. The most likely one perhaps, is by solating the oriented structure as fast as it forms, for ether applied to a mitotic figure already in existence can be seen by polarised light to solate it very rapidly. It might be expected however that a continuous solation effect of this sort would gradually deplete an energy store, and lead to increased delays; but this does not seem to be the case. It may be preferable therefore to think in terms of ether preventing the utilisation of an energy store. If, on the other hand, the experiments are interpreted in terms of a store of a non-energy carrying compound controlling mitosis (in which case mitosis itself must be supported by anaerobic glycolysis), the same difficulty does not arise. Ether might then be preventing the effect of the reservoir substance, or inhibiting the production or utilisation of anaerobic energy. The work of Gray (7) provides a starting point for further analysis of this aspect of the action of ether.

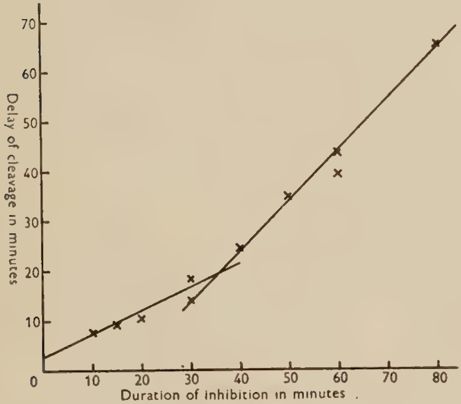
With inhibitions beginning after the critical point, namely at 45 minutes, the situation is slightly different. Once again the points for delay against duration of inhibition fall on a straight line, making an angle of about 45° with ordinate and abscissa (Text-fig. 1). It is to be expected therefore that there will be a more or less constant interval between the end of inhibition and the start of cleavage, and it can be seen from Column 4 of Table I, that this is the case. The average figure for the three experiments is 17.3 minutes, which is almost exactly the same as the interval between the critical point and the start of cleavage in the controls. The 45 minute egg is therefore reverting in some sense to the 40 minute stage. Had it merely remained blocked at the 45 minute stage, one might have expected it to take up where it left off, so that the interval between the end of inhibition and the start of cleavage would have been 57.6–45 minutes i.e. about 12 ½ minutes instead of about 17 ½ minutes.



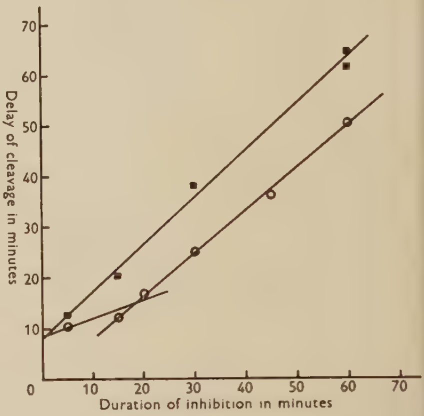
Text-fig. 2. Duration of inhibition and delay of cleavage for 2 per cent ether. Inhibitions starting at 5 minutes after fertilisation.

Since ether solates the mitotic figure, this effect is not surprising; it is only to be expected that the building up process must start afresh. It might be imagined, however, that the destruction of the mitotic figure would dissipate the reservoir substance, and cause substantial delays in cleavage as a result; but this is not the case, though it is true that eggs inhibited at 45 minutes often fail to complete cleavage when the inhibition is lifted.

The effect of 2 per cent ether raises further complications. Consider first of all inhibitions beginning at 5 minutes (Text-fig. 2). It is evident that from the very start the delays are not negligible, as they were with 1 per cent ether; they increase linearly with the duration of inhibition, being in fact equal to about half the inhibition time. It follows that ether at this concentration is halving the rate at which the reservoir fills, perhaps by depressing the rate of aerobic respiration; further information is needed on this point. To reach the critical point with an inhibition starting at 5 minutes, requires in view of this halved rate, not a further 35 minutes but about 70 minutes. A similar effect is apparent for inhibitions beginning at 20 and 30 minutes (Text-figs. 3 and 4). In these cases, 40 minutes and 20 minutes respectively are required to reach the critical point. Beyond the critical point, of course, delay increases at the same rate as inhibition, which explains the discontinuities in the graphs at these times.



Text-fig. 3. Duration of inhibition and delay of cleavage for 2 per cent ether. Inhibitions starting at 20 minutes after fertilisation.

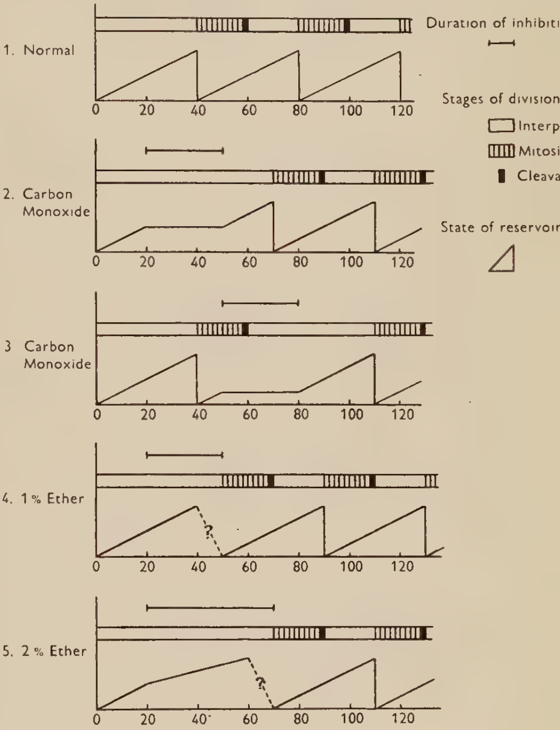


Text-fig. 4. Duration of inhibition and delay of cleavage for 2 per cent ether. Inhibitions starting at:
30 minutes after fertilisation – open circles
45 ,, ,, ,, – squares.

It will be remembered from the 1 per cent ether experiments, that the time interval between the end of inhibition and the start of cleavage is of the same order as the interval between the critical point and the start of cleavage (17 ½ minutes), implying that this concentration of ether blocks the egg at or near the critical point. An examination of Column 4 in Table II shows that the time from the end of inhibition to the start of cleavage with 2 per cent ether is fairly consistently greater than 17½ minutes. The average figure for 12 experiments when inhibition started at 5, 20 or 30 minutes, and continued up to or beyond the time required to reach the critical point, is actually 22.5 minutes. That this would be the case can of course be deduced from the fact that the lines in Text-figs. 2, 3 and 4 do not extrapolate back to zero delay at zero inhibition, but rather to a delay of about 5 minutes.

It is far from clear what this added delay implies, though in general terms it must mean that 2 per cent ether, as well as slowing down the filling of the reservoir, and blocking the building up of the mitotic figure, causes some disruption to the mechanism of mitosis which is not immediately reversed at the end of inhibition, and takes 5 minutes or so to overcome. It would be desirable to know which stage of mitosis is actually involved in this delay.

Eggs inhibited in 2 per cent ether at 45 minutes (Text-fig. 4) behave in much the same way as eggs inhibited at the same time in 1 per cent ether, and presumably revert, in the same way, to the 40 minute stage. An examination of Column 4 of Table II however, shows that, as with eggs inhibited at earlier stages, the time from the end of inhibition to the start of cleavage, is consistently greater than 17 ½ minutes. The average of five experiments is 21.0 minutes. The remarks of the preceding paragraph apply here as well.



Text-fig. 5. Diagrams to illustrate the working of the reservoir hypothesis under normal conditions, carbon monoxide and ether inhibitions. Times slightly modified for simplicity—critical point for siphoning out of reservoir, 40 minutes after fertilisation; cleavage 60 minutes after fertilisation.
1. Normal conditions.
2 and 3. Carbon monoxide.
Action: complete block to filling of reservoir.
2. Inhibition: from 20–50 minutes after fertilisation.
 Result: 1st cleavage delayed 30 minutes.
3. Inhibition: from 50–80 minutes.
 Result: 1st cleavage not delayed.
  2nd cleavage delayed 30 minutes.
4. 1 per cent ether.
Action: (a) no effect on filling of reservoir.
 (b) block to building up of mitotic figure, either by preventing siphoning out or by preventing effect of reservoir contents.
Inhibition: from 20–50 minutes.
Result: 1st cleavage delayed 10 minutes.
5. 2 per cent ether.
Action: (a) halving of rate of filling of reservoir.
 (b) block to building up of mitotic figure, as with 1 per cent ether.
Inhibition: from 20–70 minutes.
Result: 1st cleavage delayed 30 minutes.

It remains to mention the effect that inhibitions applied during one mitosis have on the subsequent mitosis. Bearing in mind that inhibition, so far from depleting the reservoir, allows it to continue filling, it might be expected that inhibition applied during mitosis (i.e. at 45 minutes), while blocking temporarily that mitosis, would allow the reservoir to go on filling; so that when the inhibition was lifted and the first mitosis completed, the reservoir would already be full and therefore able to start the next mitosis immediately. It might be expected in short that the second mitosis would follow the first with a shorter interval than usual. But to judge from a few experiments, this does not seem to be the case. Further investigation is needed however before it is worth while attempting to explain this result.

The idea of the reservoir may not be immediately easy to grasp in its entirety. In an endeavour to clarify these issues, its mode of action is shown diagrammatically in Text-fig. 5, for normal conditions, and for carbon monoxide and ether inhibitions.

DISCUSSION

Amidst the uncertainties in interpreting these experiments, there is one conclusion that stands out clearly: ether has at least two distinct effects on the process of cell division. At a concentration of 1 per cent, it blocks the building up of the mitotic figure in some way as yet uncertain, without however affecting the aerobic mechanism which induces the onset of mitosis i.e. the filling of the reservoir. At a concentration of 2 per cent a second effect becomes apparent, and the aerobic mechanism concerned with filling the reservoir is affected, functioning at only about half its normal rate.

At higher concentrations than 2 per cent, there is reason to think from a few experiments that the filling of the reservoir can be completely inhibited, but the incidence of cytolysis makes critical experiments difficult to carry out. At concentrations much below 1 per cent the filling of the reservoir is not of course affected, and the egg develops normally, though the mitotic figure may be reduced in size.

These results, together with the results of the earlier carbon monoxide experiments, leave no doubt that at about 40 minutes after fertilisation, i.e. at or soon after the beginning of prophase, a new physiological phase begins. The egg, having been dependent on an aerobic mechanism for filling up the reservoir, and unaffected by low concentrations of ether, quite suddenly becomes independent of the aerobic mechanism, so far as the completion of cleavage is concerned, but at the same time becomes sensitive to low concentrations of ether, which directly or indirectly suppress the mitotic figure.

The nature of the reservoir remains obscure. But the fact that 1 per cent ether, while causing a solation of the oriented protoplasmic structures of the cell such as the sperm aster, is without effect on the reservoir, suggests that it is not bound to any structure.

It is tempting to regard the reservoir as an *energy* store, and this naturally suggests ATP or some other energy-rich phosphorus compound as the stored substance. In this connection there is evidence that the inorganic phosphate content of the egg decreases after fertilisation at the same time as the amount of organic phosphate rises. There is general agreement however, that the amount of ATP does not vary significantly during fertilisation and division, though it appears that the amount of arginine phosphate rises sharply in the first few minutes after fertilisation (2). The time relations of this rise, however, do not seem to fit particularly well with the reservoir hypothesis.

It may be noted that the labile phosphate seems to be readily available to the cell as a whole, since it decreases during anaerobiosis (1, 3). It follows that if the energy store for mitosis were in the form of labile phosphate, it would be depleted during inhibition, with the result that the delays in cleavage would necessarily be greater than the inhibitions, in order to make up for the depletion. This however, is not the case. It may also be noted that the amounts of labile phosphate are not large, and probably only sufficient to maintain the egg of *Psammechinus miliaris* at the normal rate of metabolism for 6 minutes under anaerobic conditions (4). It seems unlikely therefore that the reservoir can consist of either arginine phosphate or ATP, but this of course does not rule out the possibility of its being some other organic phosphate.

The reservoir might alternatively act as a store of some mitotic controlling substance quite unconnected with the supply of energy; but in this case it would be necessary to suppose that the substance in question could only be formed as a result of aerobic respiration, while the mechanisms of mitosis and cleavage could function on energy derived from anaerobic glycolysis. The critical experiment, which has yet to be done in detail, would be to apply an inhibitor of glycolysis *when mitosis was already under way*. The work of Hughes (8) however, suggests that when fluoride, which is amongst other things an inhibitor of anaerobic glycolysis, is applied to tissue culture cells during mitosis, it slows down but does not necessarily stop the process.

It remains to consider the experiments in relation to other work on narcosis and cell division. It has long been realised that division, in common with various other cell activities, is more readily inhibited by narcotics than normal aerobic respiration. Urethane, for instance, blocks cell division in sea urchin eggs at a concentration which only reduces oxygen consumption to 70 or 75 per cent of normal (6). Carbon monoxide, or low oxygen tensions, on the other hand, do not block cell division until the oxygen consumption is reduced to 20 or 30 per cent of normal (9).

The results of the experiments with ether and carbon monoxide offer some explanation of why this should be. They show that the first effect of ether on cell division, and in all probability of other narcotics too, is to block the building up of the mitotic figure *at a time when its appearance is no longer dependent on aerobic respiration*. Only at higher concentrations of ether is the aerobic process concerned in filling the reservoir itself affected. The depression of aerobic respiration observed with low concentrations of urethane and other narcotics is therefore unlikely to be the immediate cause of the block to the formation of the mitotic figure.

Fisher and Henry (6) in their studies on the action of urethane and chloral hydrate on respiration and cell division in sea urchin eggs, found evidence of two aerobic respiratory pathways, probably acting in parallel, one of which was more sensitive to the narcotics than the other. They assumed that the more sensitive system was the one responsible for providing the energy for mitosis, since the less sensitive system was only affected at concentrations of narcotic above the minimum needed to produce a complete block to division. The more sensitive element they called the 'activity respiration'.

The effect of ether on respiration in sea urchin eggs has apparently not been investigated, and there would be various difficulties in doing so. But in view of the close similarity of action between all narcotics it may not be unreasonable to suppose that its action is comparable with that of urethane or chloral hydrate. Preliminary experiments with urethane suggest that its action is indeed very similar to that of ether. If so, the first effect of ether, when the appearance of the mitotic figure is just blocked, must correspond to the inhibition of the more sensitive element of respiration found by Fisher and Henry, while the second effect of ether in slowing down the filling of the reservoir, must correspond to the inhibition of their less sensitive element. As we have already seen, any respiratory inhibition accompanying the first effect is unlikely to be the cause of the block to the formation of the mitotic figure. It follows that respiratory inhibition accompanying the *second* effect, namely the slowing down of the filling mechanism of the reservoir, corresponds to the inhibition of Fisher and Henry's less sensitive element. It is this less sensitive respiration therefore, which should perhaps be called the 'activity respiration'. Further experiments however, are needed to clarify these issues.

SUMMARY

1. Earlier work on the sea urchin egg using carbon monoxide as a respiratory inhibitor, had indicated that an aerobic mechanism is responsible

for building up a store or reservoir of some sort, which siphons out at a critical point in the cycle and starts mitosis.

2. In the present experiments ether was applied to fertilised sea urchin eggs at different points in the mitotic cycle and for various lengths of time, with a view to finding out more about this reservoir mechanism.

3. The results show that ether has two distinct effects on cell division. At a concentration of 1 per cent it does not affect the aerobic mechanism concerned with filling the reservoir, but it prevents the building up of the mitotic figure, blocking the egg at the critical point referred to above. At a concentration of 2 per cent, it halves the rate of filling of the reservoir as well.

4. It is possible that the reservoir is a store of energy, but if so, it seems unlikely that the energy carrying compound can be either arginine phosphate or ATP. The fact that 1 per cent ether solates the oriented structures of the cell but does not affect the filling of the reservoir, indicates that the store is not associated with any protoplasmic structure.

I should like to thank the Director and staff of the Marine Station, Millport, Scotland, for their continuing help.

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ADDENDUM

Since writing this paper my attention has been drawn to two earlier papers that are relevant to this work. Blumenthal (*Physiol. Zool.*, **3**, 539 (1930)) showed that *Arbacia* eggs treated with cyanide at the diaster stage nevertheless completed cleavage. Runnström (*Protoplasma*, **20**, 1 (1953)) found that eggs subjected to anaerobic conditions after the dissolution of the nuclear membrane similarly completed cleavage. There is close agreement here with the present experiments.

Experimental Cell Research, **7**, *518–529* (*1954*)

THE CULTIVATION IN FLUID MEDIUM OF ORGANISED LIVER, PANCREAS AND OTHER TISSUES OF FOETAL RATS

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Since its introduction in 1929 (4), the watch-glass method for organ-culture has proved a valuable technique for the study of morphogenetic and embryological problems (2). Although its great asset lies in the fact that the tissue is explanted on the surface of the plasma clot and is then provided with free access to both nutrients and air, the solid medium possesses certain disadvantages. In the first place it limits the rate of the disposal of waste metabolites produced by the explants; the accumulation of such waste products may seriously handicap the growth and differentiation of the explant, especially when highly secretory tissue such as endocrine glands are cultivated. Moreover certain tissues have the undesirable ability to liquify the plasma clot readily, producing a pool of fluid in which the explant becomes submerged and deprived of proper air supply. In an attempt to overcome these disadvantages, Martinovitch (5) has used paired glass rods to support the explant on the top of the clot. Certain glandular tissues have been very successfully cultivated by this method, which, however, is not suitable for a highly secretory gland such as the pancreas, as it does not

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Fig. 1. Section of normal liver primordium from a 13-day rat embryo. Note the numerous blood-forming cells and scattered hepatic cells; there are no trabeculae. Formol-Zenker; haematoxylin and chromotrop. × 370.

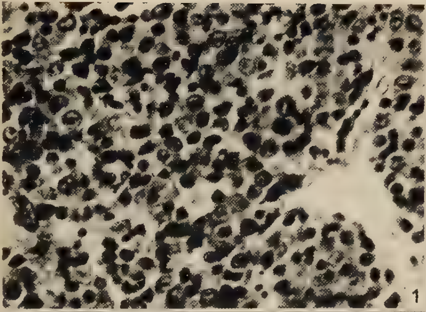
Fig. 2. Section of liver fragment from a 15-day rat embryo after 6 days' cultivation. The number of blood-forming cells has greatly decreased; trabeculae of hepatic cells are well formed and Kupffer cells (K) are present. Formol-Zenker; haematoxylin and chromotrop. × 370.

Fig. 3. Section of liver fragment from a 13-day rat embryo after 8 days' cultivation (cf. Fig. 1). Only a few normoblasts are left; the lumen of the bile duct (bd) is now clearly visible. Formol-Zenker; P.A.S. × 370.

Fig. 4. Section of the pancreatic rudiments (dorsal — dp; ventral — vp) from a 13-day rat embryo. Only a network of anastomosing tubules is present. Formol-Zenker; Bensley. × 50.

Fig. 5. Section of pancreas (dorsal primordium) from a 13-day rat embryo, after 8 days' cultivation. Note the large number of tubules, the acinar tissue at the end of all the ducts and clusters of islet cells along their course; zymogen granules are present in the acini in varying amounts. Formol-Zenker; Bensley. × 50.

Fig. 6. Section of the same specimen as shown in Fig. 5. Note the lateral budding of the islet tissue from the duct. Formol-Zenker; Bensley. × 420.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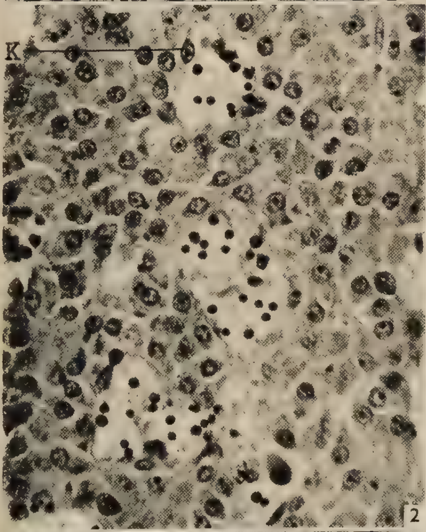

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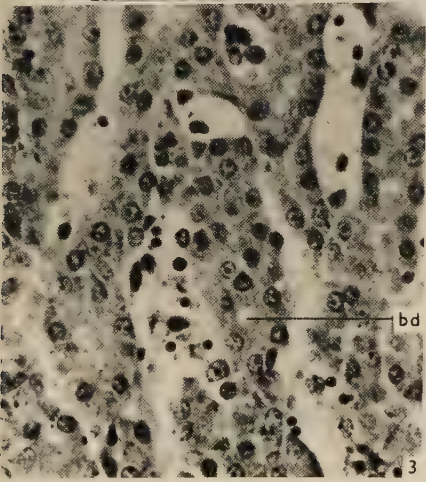

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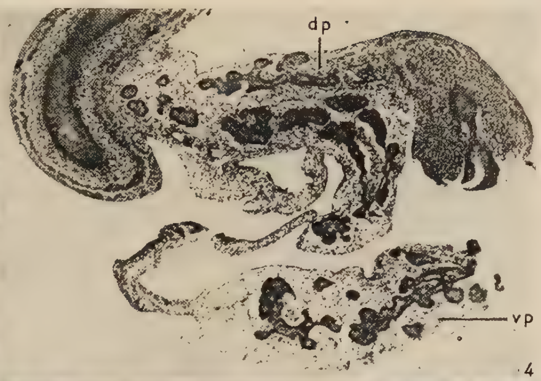

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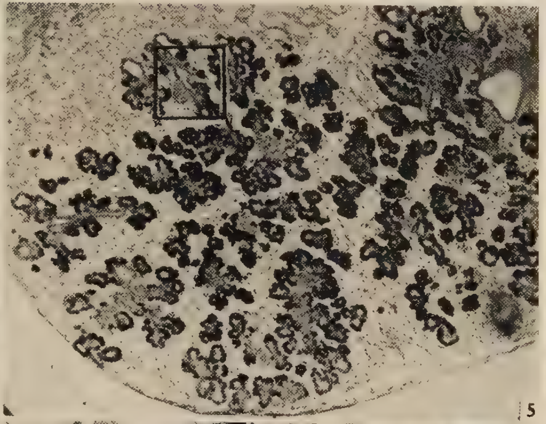
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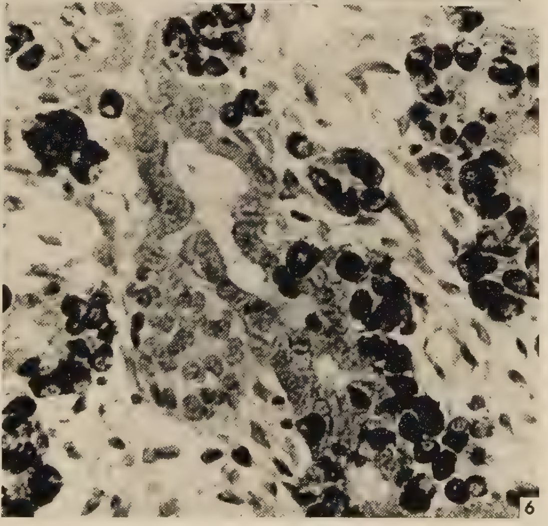
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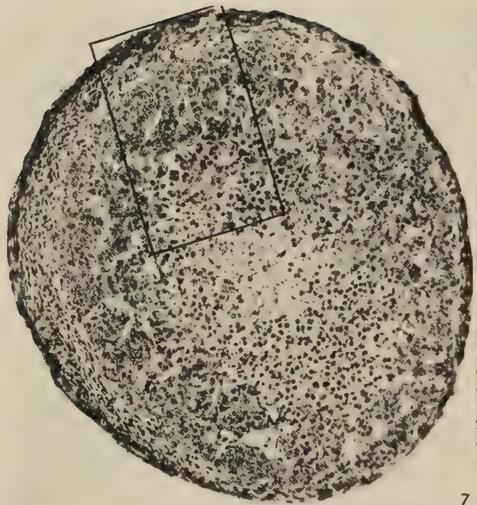
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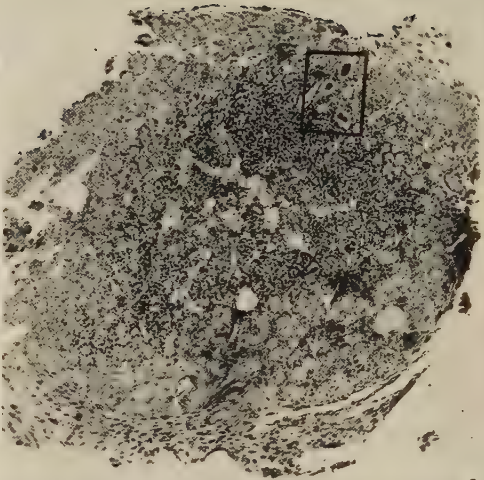
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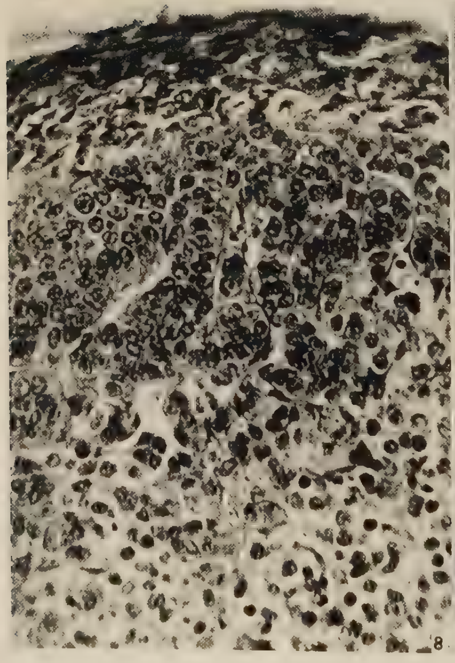
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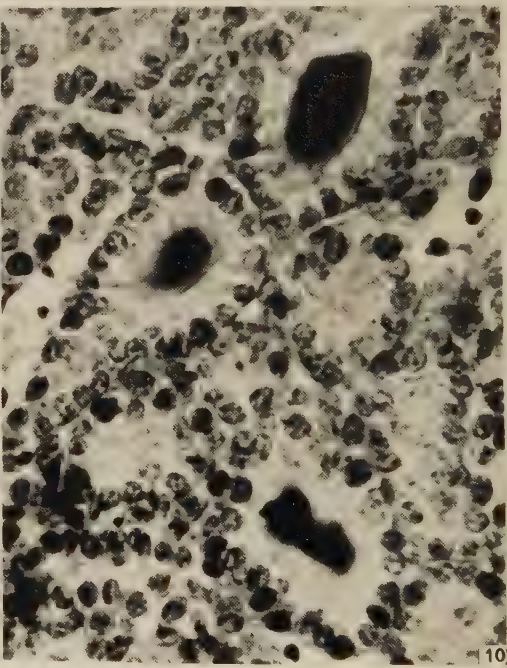
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Fig. 7. Section of the adrenal cortex from a 15-day rat embryo after 6 days' cultivation. Note the differentiation of the cortex into distinct layers of cells of different arrangements and characteristics. The central area is degenerating. Formol-Zenker; haematoxylin and chromotrop. × 140.

Fig. 7. Section of the adrenal cortex from a 15-day rat embryo after 6 days' cultivation. Note the differentiation of the cortex into distinct layers of cells of different arrangements and charac-

provide means for a sufficiently rapid diffusion of the secretory products from the immediate neighbourhood of the explant.

Many problems concerning the effects of vitamins and hormones on organ-culture are now being investigated at the Strangeways Laboratory (3, 1); in connection with this research it is planned to make biochemical observations on both the tissue and medium during cultivation; for some experiments it will also be desirable to vary the composition of the medium at intervals, if possible without disturbing the explants by subcultivation. To achieve these ends, cultivation in a fluid medium is essential.

A review of the literature shows that liquid medium was used as early as 1936, for the prolonged survival of tissue fragments in conical flask (6); this method, however, does not permit the observation of the living culture and has never been successfully used for organ cultures. No further progress along this line was made until recently Trowell (7) succeeded in growing lymph nodes on cotton wool soaked with fluid medium; he was able to maintain nodes from young rats in a healthy state for 4 days *in vitro* and to make biochemical observations on the O_2- and glucose-consumption, but he could not examine the living culture under the microscope. More recently (8) he has devised a new method in which the lymph node is explanted on lens paper resting on a piece of tantalum gauze which stands in the medium on its bent edges.

Trowell's method suggested to the author the possibility of using lens paper without the costly tantalum gauze. The lens paper used by Trowell sinks in a fluid, but it was found that some brands of thin paper float indefinitely on the liquid medium and serve as a perfect support for small explants. A method has accordingly been developed which, it is hoped, may combine the advantages of the classical watch-glass technique with those of using liquid medium. Because of the highly porous nature of the paper, nutrients and waste products are easily diffused to and away from the tissue. During subcultivation the paper with the adherent explants can be readily lifted off the fluid, washed and put on a fresh medium, so that the disturbances and damage usually involved in freeing an explant from a clot and which often gravely interfere with its growth are eliminated. The method also has

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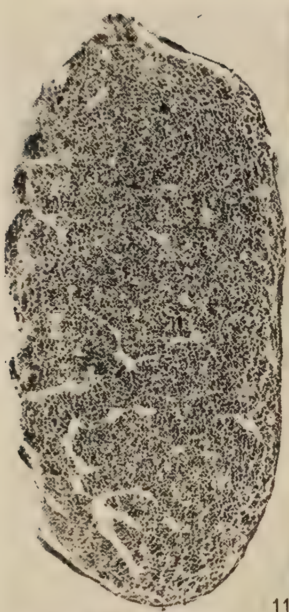
teristics. The central area is degenerating. Formol-Zenker; haematoxylin and chromotrop. × 140.
Fig. 8. Part of the section shown in Fig. 7 enlarged. Note the well-formed capsule and *zona glomerulosa*; part of the *zona fasciculata* is also present. × 420.
Fig. 9. Section of the thyroid gland from a 20-day rat embryo after 13 days' cultivation. Note the complete folliculation of the gland. The peripheral follicles are larger. Carnoy, Azan. × 70.
Fig. 10. Part of the section shown in Fig. 9, enlarged to show the follicles with colloid. × 600.



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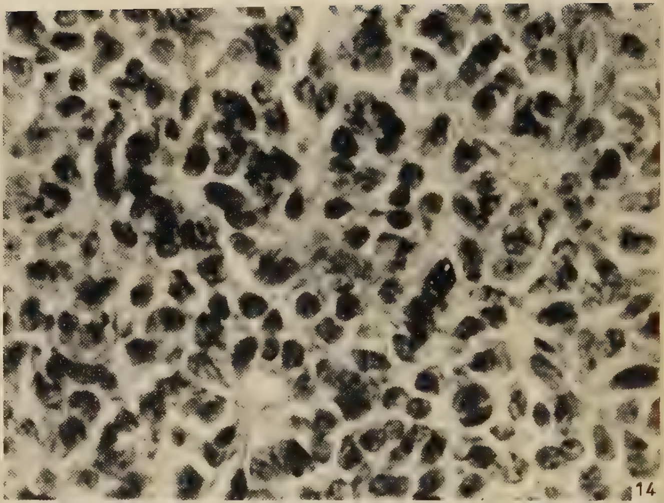

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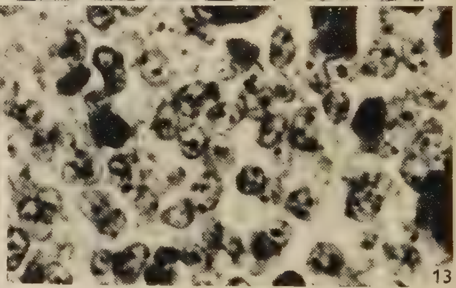

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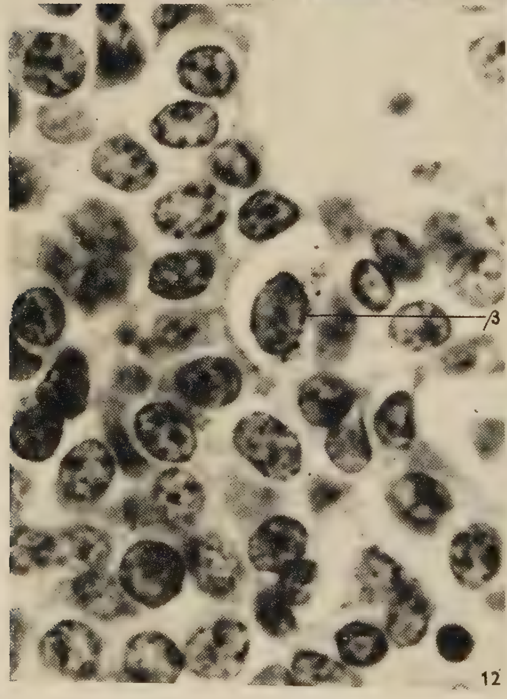

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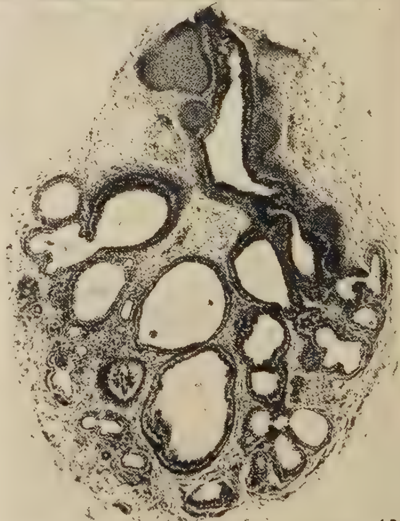

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all the practical advantages which are inherent in the use of liquid medium, such as being able to change the composition of the medium during the experiment, to take aliquots for analysis, etc.

The value of the method has been readily demonstrated by the successful cultivation of various glandular tissues from foetal rats. Tissues such as the embryonic pancreas and liver, which so far as the author is aware, have never been successfully grown as organ cultures, differentiated almost normally and grew remarkably well. It is proposed in this report to describe the details of the technique and the results obtained with different tissues.

TECHNIQUE

Preparation of culture vessels. The culture vessel is the same as that described by Fell and Robison (4) and consists of a watch-glass enclosed in a Petri-dish carpeted with a ring of wet cotton wool, the only difference being that a liquid medium with floating lens paper is substituted for the clot of plasma and embryo extract. The paper used, supplied by G. T. Gurr, Ltd., London, is cut octagonally to a diameter of about 25 mm. It is prepared by initially washing in two or three changes of ether for two hours, followed by two or three changes of absolute alcohol for two hours and finally 5 to 10 changes of glass distilled water overnight. The paper is dried in an oven (70–80° C) and sterilised by dry heat at 120–150° C for one hour.

The medium. For general use, the liquid medium is composed of equal parts of: (a) Avian embryo extract, made from 12–14 day chick embryos minced and diluted with an equal amount of Tyrode's solution; the extract is allowed to stand for twenty minutes and centrifuged for four minutes at 2000 r.p.m.

(b) Adult fowl serum. Penicillin may be added to the serum to a concentration of 1000 I.U./ml.

The proportion of the two components may be varied at will according to the requirement of the tissue. The total volume of the medium is about 0.5–1.0 ml per watch glass.

Explantation. After the embryo extract and the serum have been mixed carefully in the centre of the watch-glass, one of the octagonal pieces of lens paper is gently laid on the surface of the medium. Usually four explants, each about 2–3 mm in diameter, may be grown on one piece of paper. They are washed with Tyrode and

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Fig. 11. Section of the anterior pituitary gland from a 20-day rat embryo after 13 days' cultivation. Carnoy, P.A.S. × 90.

Fig. 12. Part of the section shown in Fig. 11 enlarged. Note the β-cells (β) with their P.A.S.-positive granules. × 1100.

Fig. 13. Section of normal spleen from a 15-day rat embryo. Formol-Zenker; Azan. × 730.

Fig. 14. Section of spleen from a 15-day rat embryo after 8 days' cultivation. Note the arrangement of cells into trabeculae and the fibrillar stroma. Carnoy, Bensley. × 730.

Fig. 15. Section of the lung from a 10-day rat embryo after 6 days' cultivation. Note the network of bronchioli; cartilage rings have been formed around the trachea. Acetic-Zenker, haematoxylin and chromotrop. × 40.

gently deposited on the lens paper by means of a medium-sized pipette, so that a small quantity of Tyrode is carried over with the tissue. This slight excess of fluid is not removed as in the classical watch-glass method, so as to facilitate the diffusion to and from the liquid medium beneath the paper. Sometimes dilute embryo extract or a little of the liquid medium itself may be used instead of Tyrode to provide an immediate supply of nutrients. The tissue is carefully flattened on the lens paper with a fine cataract knife and needle. Care is taken to ensure that only the area adjacent to the explant is moistened, as too much wetting may cause the paper to sink.

Subcultivation. This is done every 4 to 6 days and involves the thorough washing of the explants and the lens paper with Tyrode and the transfer to new medium.

The paper bearing the explants is lifted from the medium with a pair of dry, fine forceps and placed on Tyrode in another watch-glass; a little practice is required to obtain the correct angle at which to hold the paper in lifting. For each wash, the lens paper is first swirled around on the surface of the Tyrode to remove any stale medium and waste products that may attach to its lower surface. With a very fine pipette it is possible to remove from each explant first the waste liquid accumulated on the paper, and then the Tyrode underneath which diffuses upwards through the paper where suction by the pipette is applied. Eventually all the saline is removed and the paper comes to rest on the watch-glass. Drops of fresh Tyrode are then placed carefully on and around each explant from a fine pipette held very low, i.e. as near the surface of the paper as possible, since dropping the solution from any height tends to spread it over a large area of the paper and makes re-floating difficult. With proper care, as the Tyrode deposited on the tissue diffuses through the paper, the latter once again floats, can be swirled around and transferred. In lifting the paper to another watch-glass of Tyrode, it is a useful practice to "drag" it gently off the solution to ensure the cleansing of its lower surface.

The washing procedure may be repeated once or twice before the lens paper with the explants is carefully placed on fresh medium in a watch-glass.

The water soaking the cotton wool in the Petri-dish should be replenished every other day, and if the culture is highly secretory the culture vessel should be gently swirled daily to help remove any waste products that might have accumulated around the tissue.

Fixation. The explants can be fixed at any desirable intervals either after removal from the paper or directly on the paper. If different fixatives are to be used for one culture, a corner of the paper with its attached tissue may be cut off for individual fixation. If fixed on the paper with an aqueous solution, the explant can be dissected off the paper in water just before it is dehydrated. When alcoholic fixatives are used, it is best not to attempt the dissection in alcohol because of its rapid evaporation during the operation and the consequent risk of damaging the tissue through drying, but the dissection can be safely accomplished when the tissue is in cedar wood oil. In any case, before embedding, the explants should be carefully examined under the microscope and any remaining small fibres from the lens paper removed, as they cause razor marks when the tissue is sectioned.

Histological technique. Most of the tissue fragments and explants were fixed in Zenker-formol solution, some in Carnoy's fluid and some in acetic Zenker's solution. They were embedded in paraffin wax and serially sectioned at 5 μ. The sections were generally stained with Delafield's haematoxylin and Chromotrop 2R. To demonstrate granules, the Periodic Acid Schiff method (P.A.S.) was applied to sections of liver and anterior pituitary gland and Bensley's strain (crystal violet and orange G) to pancreas and spleen. Heidenhain's azan was also occasionally used.

RESULTS

Liver

When fragments of liver, about 2 mm square and 1 mm thick, from a 13- or 15-day rat embryo were cultivated by the method for 6-15 days, they continued to differentiate and grow in a surprisingly normal fashion.

There is very little difference between the liver from a 13-day and that from a 15-day embryo. At this stage it is a deep red in colour due to the fact that the sinusoids are filled with blood-forming cells. The trabeculae of hepatic cells are not yet definitely formed (Fig. 1).

During cultivation, the fragments generally spread a little and thinned out on the paper, although the amount of outgrowth was so small as to be hardly noticeable. The most striking feature was the extrusion of blood cells into the medium. As cultivation proceeded, the colour of the explants became increasingly pale and blood-coloured fluid could be seen above and around the tissue on the paper and especially in the medium beneath.

Sections of explants fixed after 6 days' cultivation showed that the number of blood-forming cells in the sinusoids was greatly reduced but there were still a number of normoblasts, some of which were degenerating. Kupffer cells were now present, lining the sinusoids (Fig. 2). The trabeculae of hepatic cells became much more clear-cut and regular than when first explanted and the cells appeared normal and healthy.

Sections of explants fixed after 8 days' cultivation indicated that the number of erythroblasts was further reduced and some normoblasts were present (Fig. 3). Pigment granules were seen in a number of macrophages which suggested active phagocytic functioning of the cells. When stained with P.A.S., some of the hepatic cells now contained a few glycogen granules, and the lumen of the bile ducts had become clearly visible. All these histological changes were very much like those that occur *in vivo*, except that they progressed more slowly. Thus the blood-forming cells disappeared much more slowly than *in vivo*, presumably due to the lack of circulation; the amount of glycogen in the hepatic cells was also considerably less than that found in liver cells in embryos of corresponding age.

After 15 days *in vitro*, there were still a few normoblasts left in the sinusoids while the macrophages were laden with pigments of haemal origin. The hepatic cells were slightly enlarged and contained glycogen; in a few the cytoplasm was vacuolated; both the sinusoids and bile ducts were abnormally dilated. It was clear that up to this stage of cultivation, the liver fragments were healthy and the cells seemed almost normal.

Pancreas

The pancreas, either the dorsal or the ventral primordium, from a 13-day rat embryo consists merely of the duct system—a network of anastomosing tubules lined by a single layer of cells. Only the ends of a very few ducts show the early sign of the formation of acinar tissue (Fig. 4). A number of buds can be seen along certain ducts but it is not possible to tell whether these are destined for the acinar or the islet tissue. The pancreas from a 15-day rat foetus has formed some acinar tissue and zymogen granules are visible in a few of the cells. No islet tissue has yet developed but buds from the tubules are more numerous.

During cultivation, the pancreas from a 13-day rat embryo remained transparent for the first three days. From the fourth day onwards, the explants became more and more opaque, the opacity spreading from the periphery towards the centre; this was due to the formation of zymogen granules in the acinar cells which could be readily observed under the microscope in the living culture. Sections of the explants fixed after 8 days' cultivation, indicated both growth and differentiation *in vitro*. The number of tubules had greatly increased and acinar tissue had developed at the end of all the ducts so that the whole system was much denser. Zymogen granules were present in varying amounts in different parts of the acini (Figs. 5 and 6). Both the acini and the lumen of the ducts were often dilated, especially in the peripheral region of the explant. A number of islets had differentiated; the presence of small clusters of islet cells along the ducts or at the points of branching but never at the ends of the ducts, suggested that they were derived from the lateral, rather than the terminal, budding of the ducts (Fig. 6). In sections stained with Bensley's method they were easily identified by their paler colour, but it was not possible to distinguish the α- and β-cells as granules failed to show up against the orange background of the cytoplasm.

When the pancreas from a 15-day rat embryo was cultivated, the results were similar. The loss of transparency of the explants was noticed almost from the very beginning of cultivation. After eight days, both the tubules and acini had greatly increased in number and their lumina were very much dilated. Zymogen granules were plentiful in the acinar cells and islet tissue differentiated during cultivation. In places, the evidence of their formation by lateral budding from the duct could clearly be observed.

After ten days *in vitro,* the islets had increased in number and size all along the side of the ducts. Mitosis was frequent and the lumina were more diluted, particularly in the periphery; the connective tissue network had become very much denser. The explants remained extremely healthy, and appeared almost normal.

Other glandular tissues

The whole primordia of the adrenal, thyroid and pituitary glands from 15–20 day rat embryos were cultivated successfully with the paper method. The results were briefly as follows.

Adrenal cortex. The rudiment of the adrenal gland in a 15-day rat embryo consists chiefly of the cortical primordium. It has a fibrous capsule, underneath which is a thin layer of small cells, and in the centre there is a fairly dense reticulum of epithelial cords. A very few sympatho-chromaffin cells are just beginning to penetrate into this cell mass. After six days' cultivation (Figs. 7 and 8), the cortex developed a well-formed *zona glomerulosa* and part of the *zona fasciculata* beneath the capsule while cells in the centre were beginning to degenerate as *in vivo.* There was no definite differentiation of the medulla, presumably due to the fact that there were not enough of the sympatho-chromaffin cells included at the time of explantation. Scattered in the explant were a few cells that resembled ganglion cells but their exact identification was difficult. It may be concluded from these results that the adrenal cortex differentiated fairly normally although the material used may have been at too early a stage to allow of the differentiation of the medulla.

Thyroid. When first isolated from a 20-day rat embryo, the thyroid had developed follicles only in the periphery while its central part consisted mainly of cell cords. During cultivation, the gland became completely folliculated and remained normal and healthy for as long as 13 days *in vitro.* Sections of the explant (Figs. 9 and 10) showed that the follicles in the periphery had enlarged and formed much colloid while those in the centre were well developed but comparatively smaller and contained less colloid. The cells all appeared normal.

Anterior pituitary gland. The anterior pituitary gland from a 20-day rat embryo is already fairly well developed. In sections it is possible to distinguish the three types of cells, i.e., the α-, and the β-cells and the chromophobe or C cells.

After thirteen days' cultivation (Figs. 11 and 12), the hypophysis remained healthy, and showed little change from when it was isolated from the body. The α-cells had large nuclei and their cytoplasm was filled with granules which, though not P.A.S.-positive, were easily discernible; the β-cells had fewer granules but they were distinctly P.A.S.-positive (Fig. 12), and the chromophobe cells were slightly smaller with paler nuclei and no cytoplasmic granules. The ratio of the three types of cells, however, seemed to have changed slightly during cultivation. There appeared to be a reduction of the number of the chromophobe cells in favour of the chromophile cells, β-cells in particular.

Spleen and lungs

Two other embryonic organs were also cultivated successfully by the paper method, namely the spleen and the lung.

Spleen. The spleen rudiment from a 15-day rat embryo (Fig. 13) grew and differentiated normally during cultivation. After eight days *in vitro*, although the cellular components were still predominantly myeloblasts and there was no differentiation into red and white pulps (which normally are not formed in foetal life), the explant developed a well-formed fibrillar stroma (Fig. 14) and some trabeculae which were not noticed when first explanted. The capillary network had multiplied and mitoses were very frequently observed.

Lung. When the lung rudiment was isolated from a 10-day rat embryo, it consisted of the trachea with two primary bronchi, one just beginning to show its 3 branches and the other, 2 branches. During cultivation, these branched dichotomously with astonishing speed and the rudiment became a dense tree-like structure with countless branches. Sections of the explant after 6 days *in vitro* (Fig. 15) showed that the trachea had developed cartilage rings and three types of branches were present: the bronchi, the bronchioles and the terminal buds—pneumonomeres. The bronchi were lined with columnar epithelium which was ciliated; the smaller branches had a single layer of epithelial cells, which were columnar in the bronchioles and cuboidal in the pneumonomeres. The whole lung had a gland-like structure similar to that found *in vivo*.

SUMMARY

A modification of the classical watch-glass method for tissue culture is described, in which the explants are grown on lens paper floating on a liquid medium. During subcultivation, the lens paper with its adherent explants

is lifted bodily from the liquid, washed in Tyrode and laid on fresh culture medium.

The method is especially suitable for growing highly secretory tissues *in vitro*, as it ensures easy disposal of waste products from the explants and minimises disturbance to the tissue during subcultivation.

Fragments of liver and pancreas from foetal rats grew and differentiated extremely well *in vitro*. Good results have also been obtained with primordia of the thyroid, anterior pituitary gland, the adrenal cortex, spleen and lung.

I wish to express my gratitude to Dr. H. B. Fell, F.R.S. and Professor F. G. Young, F.R.S. for their continuous advice and encouragement throughout this work. I am deeply indebted to Dr. A. Glücksmann for his invaluable help and criticism in the histological study. My thanks are also due to Mr. V. C. Norfield and Mr. L. J. King for technical help and to Mr. G. Lenney for taking the photomicrographs.

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AMINO ACID METABOLISM IN THE EARLY DEVELOPMENT OF THE SEA URCHIN *PARACENTROTUS LIVIDUS*

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Quantitative studies of amino acid metabolism have been restricted to some degree by the difficulty of obtaining a suitable closed biological system on which reliable determinations can be carried out. The sea urchin embryo is an excellent system for such studies. The eggs are easy to fertilize and the embryos are readily cultivated, while the number of embryos provides the ideal reference baseline for comparative studies. No nutrient is taken up during early development; embryonic structures are built up by the conversion of reserve substances already present in the egg. The amino acids are stored primarily as yolk proteins which are consumed as the embryo develops. The system is thus useful not only for studies of protein synthesis in the general sense, but also in the particular sense of a conversion of inactive yolk proteins to the specific proteins involved in embryonic development.

One could expect the reserve proteins of the yolk to be constructed in such a manner as to allow a ready and efficient conversion to the proteins involved in embryonic development. Thus, for example, amino acid sequences common to the peptide chains of many sea urchin proteins would probably also occur in yolk, and these might be taken over intact in the synthesis of new proteins. The studies reported in detail here, and briefly summarized elsewhere (26), provide some support for this hypothesis. Possibly only relatively small peptides function as such general building blocks. Perhaps such peptides can also be incorporated into the proteins of other organisms, for extracts containing them stimulate the growth of certain micro-organisms (16, 28, 3).

The author has determined the developmental changes in free amino acids and the amino acids contained in the peptides and proteins of the sea urchin *Strongylocentrotus purpuratus* (25). Large changes in the free amino acids and peptides were found to occur, whereas the proteins decreased more or less uniformly, except for a sharp decline in the late blastula. Since the changes in the protein and non-protein amino acid fractions were not found to be clearly related to one another, their interpretation was uncertain. On the other hand, if yolk-protein utilization and the synthesis of new proteins occur simultaneously, they might not be revealed by a change in the net protein. For this reason a fractionation of the proteins appeared desirable.

These studies have been continued with *Paracentrotus lividus* using improved techniques. The proteins have been separated into two fractions on the basis of solubility differences. The quantitative changes in 17 free amino acids and the amino acids of the peptides and soluble and insoluble proteins have been determined by microbiological assay of 22 stages of development and 4 'stages' of aged unfertilized eggs. The results of these studies show that relatively large changes occur in all 4 amino acid fractions. The changes are generally abrupt, of short duration, synchronous, and either complementary or supplementary to one another. It is thus possible to draw conclusions about some aspects of protein synthesis and yolk-protein degradation, and to establish the morphological stages at which these events take place. This opens the way for correlative studies of the quantitative changes in other metabolites. The investigation of changes in vitamins and growth factors is already underway at this laboratory (28, 3, 27).

MATERIAL AND METHODS

Collection of material.—The techniques for obtaining and preparing the gametes, fertilization, and culturing of the embryos were essentially the same as those already described (25), except as noted. Specimens of *Paracentrotus l.* were obtained from the Gulf of Naples in April, 1953. The eggs of 15 females, collected several hours before, were pooled. The jelly was not removed, as the eggs become too sticky and are very fragile. Fertilization was carried out in 10^{-5} M sodium periodate in sea water. The embryos were cultivated at 21° C in dilute suspensions in slowly rocked (32) 'stacking' glass trays containing 300 ml of sea water (2,500 embryos/ml). The trays containing aged unfertilized eggs were not rocked. The embryos or eggs of an entire tray were harvested quantitatively by centrifuging in 100 ml conical pyrex tubes. The material was frozen by dipping the tubes into a mixture of dry-ice and alcohol, and the frozen material was lyophilized in the same tubes.

Preparation of extracts.—To fractionate the proteins, the lyophilized material was extracted in 0.1 M KCl–0.011 M HAc. A dilute salt extraction is necessary to minimize any effect of the small but variable quantity of sea water salts which are collected with the embryos. If 0.1 M KCl is used alone, the supernatants obtained after centrifugation at ordinary speeds are deeply clouded, whereas in 0.011 M HAc, crystal clear or only very faintly opalescent supernatants are obtained. The material was first transferred from the collecting centrifuge tubes to a glass-tube homogenizer. After homogenizing, the suspensions were transferred to 45 ml pyrex-glass conical centrifuge tubes possessing a pouring-lip. To obtain quantitative transfer, the various vessels were rinsed with aliquots of the solvent. The material was extracted thrice for 24-hour periods in 40, 20 and 10 ml volumes, respectively, of the ice-cold solvent.

This triple extraction quantitatively removes components soluble in this solvent, as judged by the ninhydrin and brom-phenol-blue (12) tests. To sediment the residues, the tubes were centrifuged at 1490–2400 g RCF in the International Refrigerated Centrifuge (head 269) for 1 to 3 hours until clear supernatants were obtained. The supernatants were decanted into 250 ml pyrex beakers and lyophilized. The residues were transferred quantitatively to a new set of centrifuge tubes using 5–10 ml of water and rinsing carefully. After a second lyophilization, the material was ready for the extraction of free amino acids, peptides and other dialyzable components.

In the study of *Strongylocentrotus p.* (25) the separation of amino acids and peptides from the proteins was effected by successive extractions in 75 and 50 per cent ethanol. The author has found, however, that small amounts of sea urchin proteins dissolve in 50 per cent ethanol and that this solvent does not quantitatively remove free amino acids. For these reasons the ethanol extraction has been abandoned. Instead, an acetone-HCl solvent which extracts all dialyzable components but no protein and only small amounts of KCl has been developed. To satisfy all 3 conditions, 5 per cent 0.5 N HCl in acetone proved to be the optimum solvent composition.

The residues were first maintained for several days at 20° C in 5 ml petroleum ether to facilitate denaturation of the KCl-HAc soluble proteins and dissolve lipids. After centrifuging, the petroleum ether was decanted (only faint traces of amino acids are lost) and the residues were suspended in 1.5 ml of 0.5 N ice-cold HCl and subsequently 28.5 ml of acetone was added by squirting through a syringe (to provide mixing). The suspensions were allowed to stand at 3–5° C for several hours, centrifuged and decanted into 250 ml pyrex beakers. This extraction was carried out 6 times. The acetone was removed from the combined supernatants by evaporation under an air-jet, the 0.5 N HCl by repeated lyophilization. The final 'acid free' residues, which contain the dialyzable components of the original KCl-HAc extracts, were taken up in 15 ml of distilled water and stored at $-20°$ C.

The soluble- and insoluble-protein residues were hydrolyzed (in the centrifuge tubes) in 2 ml of 6 N HCl at 120° C for 10 hours. Aliquots of the non-protein extracts were hydrolyzed in 4 N HCl at 120° C for 8 hours. The HCl was removed by vacuum desiccation at 5 10 mm Hg over NaOH pellets, followed by repeated lyophilization. The hydrolyzed residues were taken up in 15 ml of distilled water, filtered to remove humin, and stored at $-20°$ C.

Microbiological assay.—The semi-micro method already described (25, 31) was used with the modifications indicated below. All media except those for assay of alanine and serine were obtained from H. M. Chemical Co., Santa Monica, California, U.S.A. (see (25) for references to the composition of these media). For serine and alanine the media and modifications of Steele *et al.* (47) and Sauberlich and Baumann (46), respectively, were employed. The medium for glycine was supplemented (2) with 0.2 ml tween 80, 100 γ thymidine, 2 γ leucovorin (omitting reticulogen), 0.4 g sodium citrate, 10 mg aspartic acid, 10 mg asparagine and 20 mg glutamine per 100 ml of double-strength medium. The same supplement, but omitting aspartic acid and asparagine, was added to the media for alanine and serine. *Leuconostoc citrovorum* (8081) was used to assay glycine. *Leuconostoc mesenteroides* P-60 was used for arginine, histidine and lysine. To avoid carmelization, the sugar (powder no. 2 of the H. M. Chemical Co. medium) was sterilized separately and added aseptically to the autoclaved medium (except in the threonine assay). A final assay volume of 0.4 ml was used. Aliquots were dispensed into 5.5 × 1cm test tubes with a British 'Summit' 0.5 ml syringe (20-gauge needle) modified as a pipette to deliver 0.2 ml.

Stock cultures were carried on Difco micro assay culture agar. The cultures were refreshed monthly by 24- to 48-hour incubation on the litmus milk composite recommended by Banhidi *et al.* (4) using 0.5 ml of Parke Davis liver concentrate in place of Heptomin and supplementing (2) with 2 g of glucose and 1 g of agar per liter. The inocula were cultured on Difco micro inoculum broth, incubating for 16 hours at 37° C in the cases of *L. citrovorum*, *L. fermenti* 36 and *L. mesenteroides*, and 24 hours for *L. arabinosus* 17-5 and *L. brevis* (8287). *L. citrovorum* was refreshed on litmus milk before preparing an inoculum for the glycine assay. The media and samples were sterilized at 10 pounds pressure for 10 minutes, except in the free glutamic acid assay where 15 pounds for 20 minutes was employed to insure more complete conversion of glutamine to pyrrolidone-carboxylic acid, and in the free cysteine assay where the samples were merely steamed for 15 minutes. The sugar and inoculum (1 ml/20 ml medium) were added to the medium together before dispensing (in a sterile room) to the samples and standards.

Although microbiological assay is an excellent method for the quantitative determination of amino acids in hydrolysates, it is slightly less reliable for free amino acids in tissue fluids and organ extracts. In the latter case, the presence of peptides containing amino acids which can be utilized by the assay micro-organisms will lead to assay values for the free amino acids which are too high. Amino acids contained in peptides are usually less active than the corresponding free amino acids (cf. 10). *L. casei* and *L. delbrueckii* 3 were not used because peptides are exceptionally stimulatory for them (13, 30). On the other hand, Dunn *et al.* (cf. 13), using essentially the same media and bacteria used here, found the free amino acid activity of partial hydrolysates to be proportional to the degree of completeness of hydrolysis.

The level of peptides (other than certain species such as glutathione and carnosine) in cell extracts is believed to be extremely low (cf. 8, 10). However, using ion-exchange and charcoal chromatography it has been possible to demonstrate the existence of many peptides in extracts of sea urchin eggs (29). In the present work, peptide (or bound) amino acids have been estimated from the difference in free amino acids before and after hydrolysis of the protein-free extracts. Although this method has its limitations, it appears to be the best available for comparative studies on a micro scale.

Practical limitations prevented assaying the amino acids at more than one concentration level. The results of test assays at more than one level show excellent agreement. The curves for the various amino acid fractions are believed to accurately depict the relative concentration changes in development, although the actual quantitative values have not been the object of intensive study.

All the techniques used in this study have been designed and adapted to best detect the *changes* in metabolites which occur in development. Thus, the embryos are dispensed initially, cultured, and harvested in as nearly equal numbers as is practically possible. There are no ancillary weighings or nitrogen or other baseline determinations such as have generally been used in the past to relate results quantitatively. All operations are carried out on equal aliquots as nearly simultaneously as practicable and with a minimum of transfers. Aliquots of extracts representing equal numbers of embryos from the entire developmental series are assayed simultaneously. All 4 fractions were generally assayed at the same time. Such experimental procedures are vital if small developmental changes are to be detected and if the entire spectrum of changes is to be undistorted.

RESULTS AND INTERPRETATIONS

The developmental changes in the molar sum of 17 amino acids and free glycine are shown in Fig. 1 together with the time of development or aging and the morphological stage represented by each point. The curves for the individual amino acids of the soluble and insoluble proteins are given in Fig. 2, those for the free amino acids, the sum of 6 peptide amino acids (including glutamine and asparagine) and the total individual amino acids, in Fig. 3. The latter curves (Figs. 2 and 3) are best compared when represented upon the basis of percentage of the value in the unfertilized egg (with all ordinates to the same scale). The concentrations of the amino acids in the unfertilized egg and their molar abundances relative to glycine are listed in Table. I.

The developmental changes in the amino acid fractions are seen to be abrupt and of relatively short duration. Since the changes in the protein and free amino acid fractions are usually complementary to one another, the obvious conclusion to be drawn is that these changes are caused by the degradation and synthesis of proteins to and from free amino acids. It is logical to assume that the proteins being degraded are the reserve yolk proteins, whereas those being synthesized are new functional proteins.

It is of great interest that these degradative and synthetic activities are more or less temporally dissociated, so that they follow one another in distinct waves. While this may not be the general pattern of protein metabolism in yolk-nourished embryos, its existence in the sea urchin is a strong indication that the metabolic organization of protoplasm required for these functions cannot serve simultaneously for both.

TABLE I

Amino acid content of the unfertilized egg (10^{-13} moles/embryo).

	Free	Peptide	Soluble protein	Insoluble protein	Total	Relative molar values (this study)	Relative molar values (15)
Alanine	155	—	132	450	737	24.5	15.2
Arginine	64.3	—	62.2	249	375	12.5	11.6
Aspartic Acid	44.8	88.7*	151	403	688	22.8	25.6
Cysteine	4.26	19.2	21.2	106	150	5.00	6.67
Glutamic Acid	74.8	60.2†	168	406	709	23.6	23.6
Glycine	2,590	—	62.8	356	3,010	100	100
Histidine	8.52	9.86	18.3	79.2	116	3.85	4.35
Isoleucine	79.2	—	82.5	250	412	13.7	16.4
Leucine	109	—	113	404	625	20.8	20.0
Lysine	73.9	—	67.3	256	397	13.2	14.2
Methionine	29.3	—	30.2	162	222	7.36	6.10
Phenylalanine	20.6	8.65	40.2	154	224	7.44	8.70
Proline	48.9	—	60.7	225	334	11.1	10.9
Serine	188	—	85.7	298	571	19.0	20.6
Threonine	59.9	—	88.0	228	376	12.5	13.5
Tyrosine	12.4	8.92	33.4	112	167	5.53	5.50
Valine	78.0	—	111	328	517	17.2	24.8

* Includes free asparagine.
† Includes free glutamine.

Recent studies (17) have shown that intense mitochondrial development in sea urchin embryos occurs in different regions at different times. The pattern changes continuously but the appearance of mitochondria in an organ rudiment seems to be closely correlated with the period of its most intense differentiation. Ries' (41) studies on *Ciona* suggest a close connection between the distribution of mitochondrial germs and the site of elaboration of structural proteins, while Gustafson and Lenicque (17) have concluded that mitochondrial enzymes are involved in the synthesis of structural proteins (see Haurowitz (21) for other references to mitochondria as protein forming units). It has also been observed that intense yolk-granule degradation occurs at certain times and centers in the sea urchin embryo (14), and it is known that yolk utilization is closely coupled with amphibian morphogenesis (cf. 7). It thus appears highly probable that these cycles of mitochondrial activity and yolk-granule degradation are visible mani-

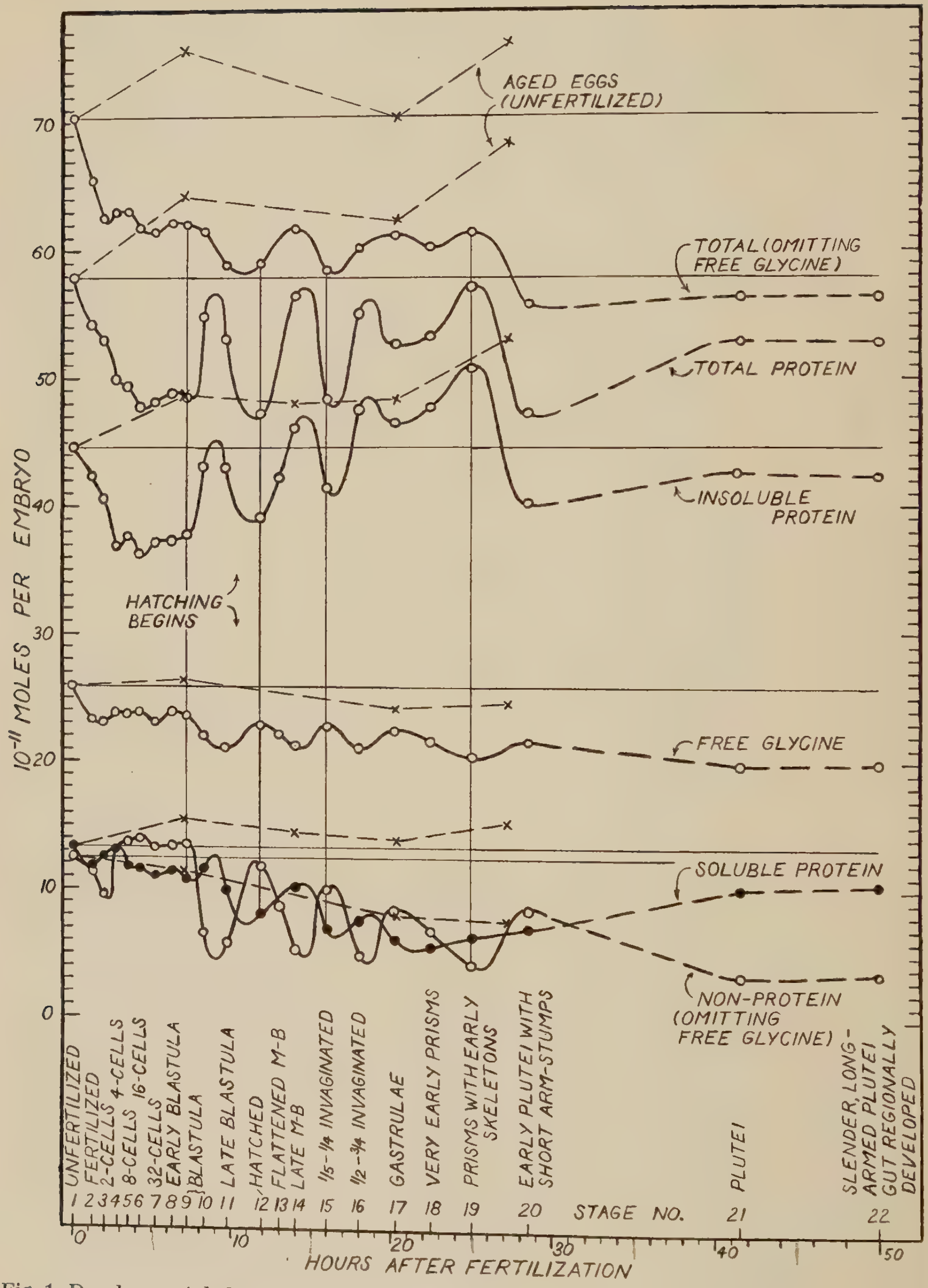

Fig. 1. Developmental changes in the molar sum of 17 amino acids (see Table I) and free glycine.

festations of which the cyclic changes in the amino acid fractions are one of the biochemical counterparts. The occurrence of cyclic changes in the amino acid fractions indicates that in this batch of embryos, or in *Paracentrotus l.* generally, there is a high degree of correlation between the active phases of the metabolic centers of the embryo. In the absence of close correlation, the overlapping of the changes accompanying protein degradation in some centers would tend to cancel those accompanying the synthesis of new proteins in others, insofar as the net observable transfer between amino acid fractions is concerned. The pattern of overlapping may vary from species to species and even intraspecifically, for it is well known that in some batches of sea urchin embryos the development of certain organs may be retarded or accelerated. It is already known (25) that the pattern itself, at least the early part, can shift relative to the morphological stages, depending upon the condition of the eggs at the time of fertilization. Developmental changes in free amino acids have been detected in amphibian embryos (31, 9) and in chick embryos (51). There is thus some reason to believe that yolk-protein degradation and new protein synthesis are at least partly dissociated in these forms (see p. 551).

Proteins.—The soluble and insoluble proteins show distinctly different developmental changes (Figs. 1, 2, 4, 5), although the main cyclic variations occur in both. The individual amino acids of both fractions show almost identical changes but there are minor qualitative differences and considerable quantitative ones. These differences are to be expected, for both fractions are surely heterogeneous and of changing composition. Only 23 per cent of the proteins in the unfertilized egg is soluble in the KCl-HAc solvent. This figure goes as low as 10.5 in the early prism (stage 18) and as high as 27.8 in the 4-cell stage, while in the 50-hour pluteus the value is 24.7. The proteins whose solubilities change on freezing and thawing (37) and as a result of fertilization (37, 38, 35) appear to be insoluble.

If the cyclic changes in the protein fractions are caused by successive waves of yolk-protein breakdown and new protein synthesis, it should be possible to obtain quantitative information about these processes by analysis of the changes occurring. Thus, the monotonic curves formed by summing the increasing phases of the protein curves should give a rough measure of the synthesis of new proteins, while those formed by summing the decreasing phases should approximate yolk-protein consumption. It is clear, however, that if synthesis and degradation within either fraction were to overlap appreciably, the record obtained by this analysis would be incomplete.

The monotonic curves formed in this way (all 17 amino acids weighted

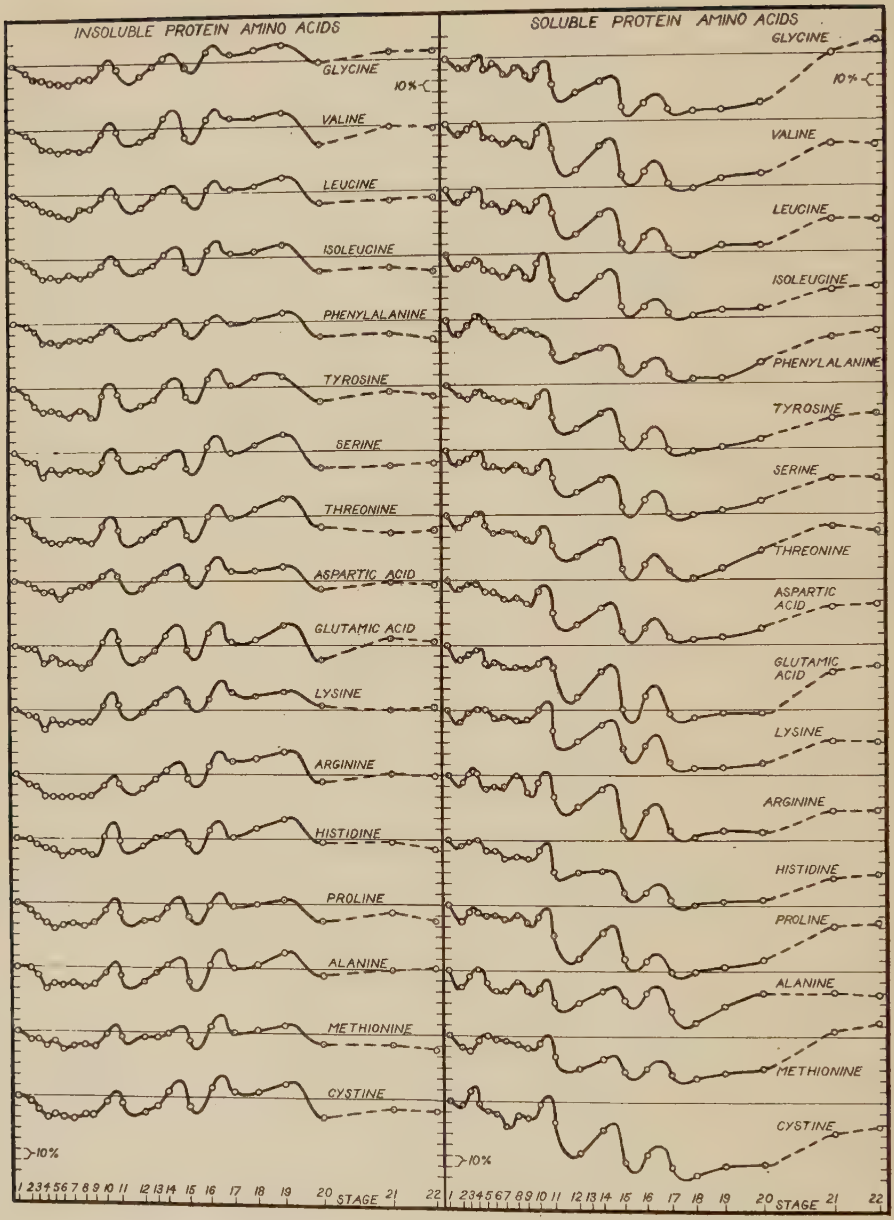

Fig. 2. Developmental changes in the amino acids of the insoluble and soluble proteins expressed as the percentage of the value in the unfertilized egg (the continuous horizontal gridlines are spaced at 60 per cent intervals).

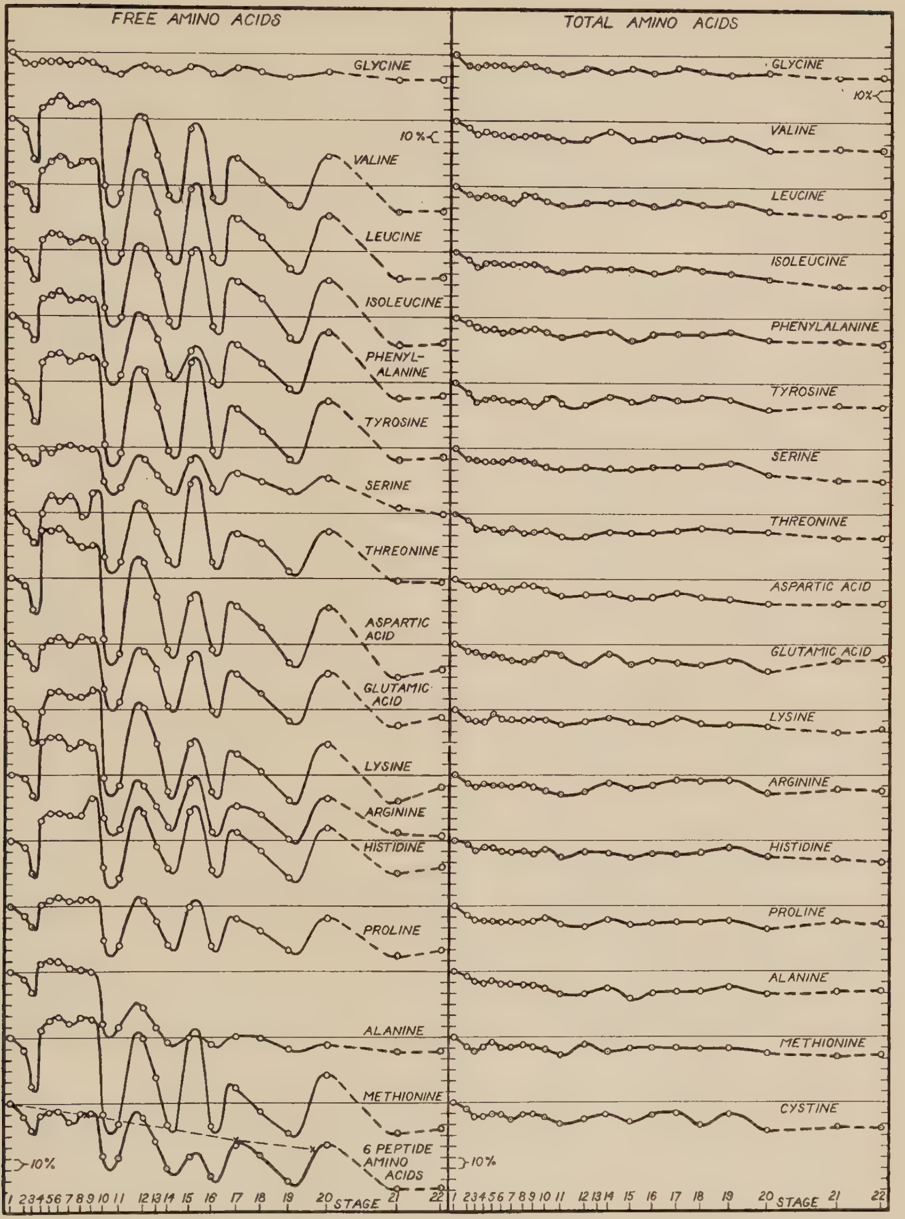

Fig. 3. Developmental changes in the free amino acids (and 6 peptide amino acids) and total amino acids. (The values are expressed in the same way and to the same scale as those in Fig. 2.) See Fig. 6 for the curve for free cysteine.

equally and averaged) are shown in Fig. 4 and the changes are further depicted in Fig. 5. The sum of the decreasing phases of the soluble protein curve attains to 95.1 per cent of the value in the unfertilized egg, while the increasing phases add up to 75.1 per cent. Thus, it appears that almost all the soluble proteins in the unfertilized egg are yolk proteins, while all those in the pluteus are new embryonic proteins. The consumption of soluble yolk proteins appears to be completed by stage 18. This would account for the fact that there is no drop in the soluble proteins corresponding to the major decline in insoluble proteins between stages 18 and 19. It is also consistent with the fact that the soluble proteins increase monotonically after stage 18. On the other hand, the consumption of insoluble yolk proteins amounts to only 66 per cent of the insoluble proteins in the egg, with the new insoluble proteins attaining to 58.8 per cent. This suggests that at least 66 per cent of the insoluble proteins is yolk proteins and at most 34 per cent is structural and other non-yolk proteins.

The differences revealed by the above analysis are of the character one would expect. The structural proteins and cell particulates would be expected to be insoluble in the solvent used, so that part of the insoluble protein fraction of the egg would consist of its functional apparatus. As a result, we should not expect the percentage consumption of yolk from this fraction to attain to that of the soluble fraction. Some insoluble yolk proteins doubtless remain in the 50-hour pluteus but a large fraction of the 34 per cent in question is probably non-yolk proteins.

The total molar decrease of protein amino acids to the 50-hour pluteus is 10 per cent, that of all amino acids, 20.8 per cent. The corresponding figures for *Strongylocentrotus p.* (to the early pluteus) are 16.5 and 19.1, respectively. From stage 10 to stage 18 (Figs. 1, 2, 4) there is a considerable transfer of amino acids from the soluble protein fraction to the insoluble one. This doubtless represents synthesis of new insoluble proteins at the expense of amino acids derived from soluble yolk proteins. After stage 18 there is a marked increase in new soluble proteins, apparently rich in methionine and glycine (see Fig. 2 and Table II).

The fact that the consumption of soluble yolk proteins amounts to 95.1 per cent of the soluble proteins in the egg indicates, insofar as this analysis is valid, that there is no appreciable overlap of synthesis and degradation of soluble proteins. The value of 66 per cent for the insoluble protein fraction does not preclude the possibility of considerable overlapping. The main waves of protein degradation and synthesis occur simultaneously in both fractions, but there are at least 5 periods when degradation in one fraction

Table II

Protein composition (percentages of the molar sum at the respective stages).

	Stage					
	Aged eggs (7 hours)		Unfertilized eggs		50-hour plutei	
	Soluble	Insoluble	Soluble	Insoluble	Soluble	Insoluble
Alanine	8.95	10.55	9.90	10.1	9.81	10.8
Arginine	4.83	5.47	4.68	5.58	4.02	5.86
Aspartic Acid	11.8	8.30	11.4	9.03	11.2	9.15
Cysteine	1.82	2.30	1.60	2.37	1.58	2.24
Glutamic Acid	12.8	9.54	12.7	9.10	13.0	9.93
Glycine	4.73	7.93	4.73	7.97	6.76	9.05
Histidine	1.44	1.85	1.38	1.77	1.20	1.71
Isoleucine	6.45	5.47	6.21	5.60	5.38	5.05
Leucine	8.71	9.10	8.51	9.05	7.49	8.92
Lysine	4.98	6.13	5.07	5.74	4.53	6.22
Methionine	2.30	3.49	2.28	3.63	3.27	3.31
Phenylalanine	3.04	3.26	3.03	3.46	3.38	3.02
Proline	4.57	4.72	4.57	5.03	4.86	4.70
Serine	6.58	6.80	6.45	6.68	5.98	6.21
Threonine	6.36	5.02	6.63	5.11	7.10	4.65
Tyrosine	2.44	2.52	2.51	2.50	2.31	2.41
Valine	8.22	7.36	8.34	7.35	8.15	6.73

overlaps with the incorporation of new proteins into the other (stages 2 to 4, 4 to 5, 6 to 7, 17 to 18, and 19 to 20). This shows that both processes can occur at the same time, although they probably take place in different parts of the embryo. In this connection it has been observed that when mitochondria are active in any region of the embryo, the yolk granules appear to be confined to some other region (14). If the total protein had been assayed without fractionation, these details would not have been detected because of overlapping. It is possible that a mere change in solubility accounts for some of the complementary changes in the protein fractions, but it does not appear likely that such a process plays a significant role.

The amino acid composition of the proteins in the unfertilized egg before and after 7 hours of aging and in the 50-hour pluteus is given in Table II. The soluble proteins are richer in acidic amino acids, the insoluble ones in basic amino acids. This might be expected, for the insoluble fraction contains the nucleoproteins, which are not soluble in 0.1 M KCl. The cysteine content of the insoluble proteins is higher, consistent with the importance of cysteine in structural proteins.

Free amino acids.—The qualitative pattern of changes in the individual free amino acids is highly uniform (Fig. 3), although rather large quantitative differences occur. Except between stages 1 and 3 (see pp. 547–548) the changes are synchronous with, complementary to, and of the same order of magnitude as those in the total protein amino acids (Fig. 1). The drain and supply of free amino acids by protein synthesis and degradation is of such magnitude that it determines the qualitative pattern of their changes. On the other hand, the data indicate that extensive amino acid interconversions do not occur.

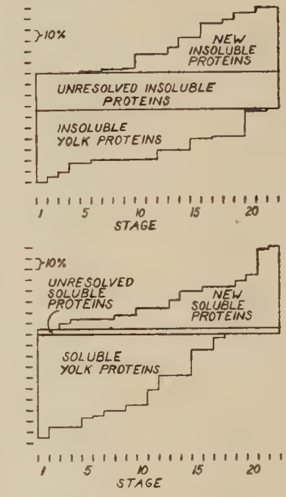

Fig. 5 (right). Diagrammatic representation of the changes in the yolk proteins and new embryonic proteins. The values are expressed as the percentages of the total protein in the respective fractions of the unfertilized egg.

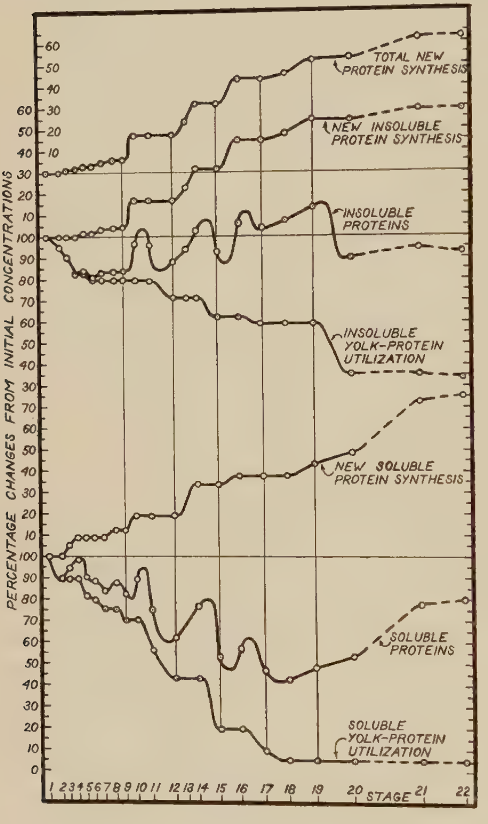

Fig. 4 (left). Average percentage changes of the insoluble and soluble protein amino acids from their values in the unfertilized eggs (all amino acids are weighted equally). The yolk-protein and new-protein curves are formed as described on p. 537.

Glycine is by far the most abundant free amino acid (Table I), comprising 71.2 per cent of the free amino acids determined in the unfertilized egg. The large amount of free glycine is generally regarded to be of osmotic significance (cf. (25) for discussion and references). Because of the high concentration of free glycine, the magnitude of the developmental changes induced in it by protein synthesis and degradation is very much less than for other free amino acids (Fig. 3). Two other low molecular weight amino acids, serine and alanine, are next most abundant at 5.15 and 4.26 per cent, respectively. Present in lowest concentration are histidine (0.234 per cent) and cysteine (0.117 per cent).

The concentration of free cysteine in the egg is unusually low and its developmental changes are of larger than average magnitude (Fig. 6). The relative developmental concentrations of other free amino acids show varying divergences from the average, histidine being generally higher, and alanine, lysine and methionine, lower. The total molar loss of free amino acids (excluding glycine) is 70.3 per cent, that of free glycine is 23.2 per cent (28.5 per cent in *Strongylocentrotus p.*).

Peptides.—Curves for developmental changes in peptide-contained (or bound) amino acids have been obtained for aspartic acid, cysteine, glutamic acid, histidine, phenylalanine and tyrosine (Fig. 6). The curve for glutamic acid represents both peptide glutamic acid and free glutamine. Asparagine is also present in the sea urchin embryo (25). It is included in the determinations of peptide aspartic acid, for since asparagine has a much lower growth stimulating effect on *L. mesenteroides* at the level assayed (18), it is detected only after conversion to aspartic acid by hydrolysis.

The curve for peptide cysteine may give a fair representation of the changes in glutathione, for glutathione has very little cysteine activity for *L. mesenteroides* (42). In some preliminary determinations of glutathione by the method dependent upon its coenzyme activity, the changes found (aged eggs and stages 1 to 4) were qualitatively the same as for peptide cysteine (1).

In the assays of all other amino acids, the differences between the determinations before and after hydrolysis were not such as to yield values for peptide amino acids. In some cases there was frank hydrolytic loss (the conditions of hydrolysis were somewhat severe) while in others the differences were insignificant.

In all 6 cases where assays for peptide (and amide) amino acids have been obtained, the values are of the same order of magnitude as those for the corresponding free amino acids (Fig. 6). In the apparent absence of certain amino acids in the peptide fraction, this might be interpreted as indicating the presence of only relatively small peptides. On the other hand, the curves for peptide and amide aspartic and glutamic acids probably

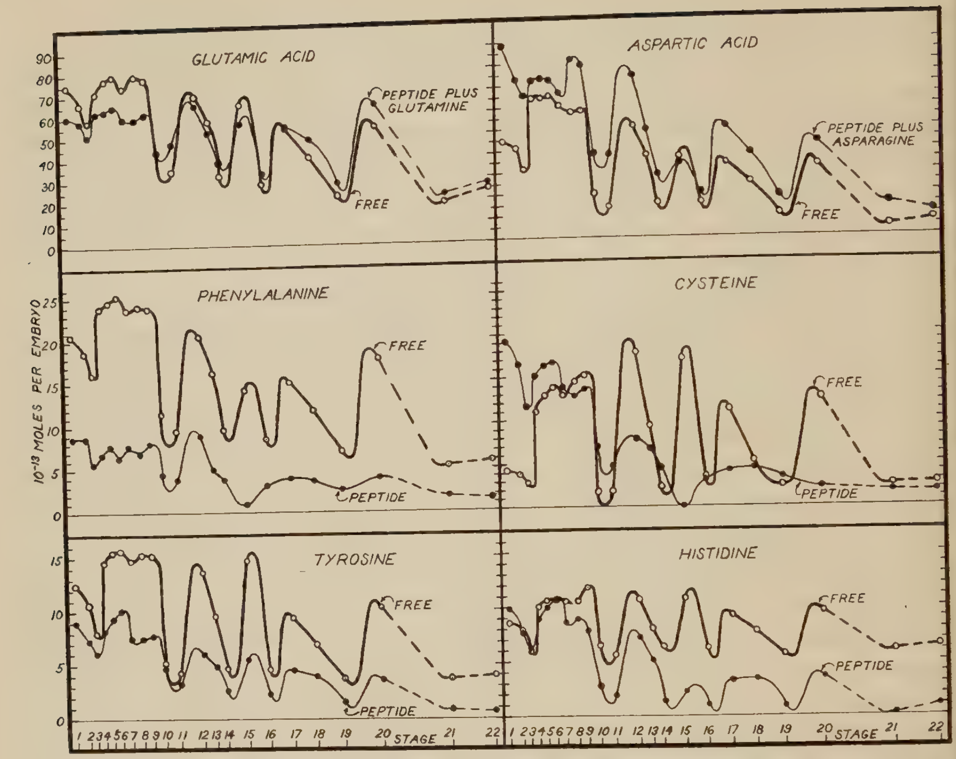

Fig. 6. Molar developmental changes in 6 peptide-contained amino acids.

chiefly represent asparagine and glutamine. It is doubtless no coincidence that the other amino acids for which peptide curves have been obtained are the 4 amino acids present in lowest concentrations in the free form (Table I). The concentrations of peptide amino acids are probably much more uniformly distributed than those of free amino acids. Thus, the success of the difference method of peptide assay for these amino acids is most likely attributable to the fact that only in these 4 cases is the amount of the peptide-contained amino acid of about the same magnitude as that of the free amino acid, and the increment after hydrolysis great enough to be detected.

A considerable amount of peptide glycine was found in *Strongylocentrotus p.* (25). There is also some evidence of peptide glycine in *Paracentrotus l.* but the differences between the values for free glycine before and after hydrolysis of the non-protein fraction are small and, in fact, they are generally higher for the unhydrolyzed extracts. An apparent decrease of glycine is also found after hydrolysis of urine (36). A simple mixture of glycine dipeptides is 20 per cent more active for *L. citrovorum* than the equivalent amount of free glycine (36). Stimulation by glycine peptides present in the extracts of sea urchin embryos probably accounts for the failure to detect them by microbiological assay. The curve for free glycine (Figs. 1, 3) represents the average of the very similar curves obtained before and after hydrolysis of the non-protein extracts.

On the whole the pattern of changes in the peptide amino acids is similar to that for the free amino acids (Figs. 3 and 6). The peptide amino acids, however, do not attain to such high concentrations in later development (relative to the unfertilized egg) and the concentrations at stage 15 are relatively low for cysteine, histidine, and phenylalanine. The total molar loss of peptide (and amide) amino acids to the 50-hour pluteus is 77.7 per cent.

Like those of the free amino acids, the changes in the peptide amino acids are generally complementary to those of the proteins. It thus seems reasonable to conclude that these peptides primarily represent either intermediates in the degradation of yolk proteins and the synthesis of new proteins to and from free amino acids, or units which are transferred intact from yolk proteins to new proteins, or both. The fact that the distribution of concentrations of peptide amino acids differs from that of free amino acids would appear to favor the interpretation that transfer of intact peptides is occurring. The growth factors occurring in the sea urchin embryo (16, 28, 3) are believed to be peptides, and they also undergo changes complementary to those of the proteins (28). They stimulate the growth of a number of micro-organisms (3). Since their stimulating property may reside in the ability of the micro-organisms to incorporate them intact into their proteins, the former finding also supports the view that intact transfer occurs.

Aged eggs.—When unfertilized eggs are allowed to stand in sea water, relatively large changes take place in the amino acid fractions (Figs. 1, 3). After 7 hours (9 to 10 hours after removal from the ovary) the molar sums of the amino acids of insoluble and soluble proteins have increased to 109 and 117 per cent, respectively. In the next 13 hours the insoluble proteins change very little while the soluble proteins decline to about 105 per cent. A sharp increase to 119 and 115 per cent occurs in the last 7 hours. At the same time the free and peptide amino acids decline continuously to only 62.9 and 57.6 per cent, respectively, of their values in the 'fresh' unfertilized egg.

At the time the eggs are spawned artificially (by placing the ovaries in sea water) they are not necessarily in natural spawning condition but may be under-ripe to varying degrees, depending upon the season and other environmental and temporal factors. The accumulation of yolk by the egg probably occurs throughout the ripening process. The finding that the egg proteins increase while the free amino acids and peptides decrease after removal from the ovary indicates that the synthesis of yolk proteins continues to take place. Since this is probably a continuation of the process which takes place in the ripening egg *in situ*, it indicates that the proteins of the egg are synthesized by the egg itself, the ovary merely supplying the amino acids (there is cytological evidence for the synthesis of yolk proteins by frog oocytes, cf. (7)). Whether the ovary also supplies the peptides which are present, or whether they are synthesized by the egg, cannot be concluded from the data, but the former process would appear to be favored.

The author has already shown (25) that in eggs from freshly collected *Strongylocentrotus p.* the free amino acid content is high and shows an initial drop after fertilization, whereas their content is low and increases after fertilization of eggs obtained from animals collected in season but kept for weeks under conditions which inhibit spawning. It now appears that a high content of free amino acids is characteristic of eggs in which intense protein synthesis was proceeding at the time of removal from the ovary, whereas a low content is an indication that this synthesis was low or had ceased, free amino acids (and possibly peptides) were no longer being taken up from the ovary, and the eggs were ripe and ready to be spawned.

Runnström (44) has observed that the fertilizability and development of unripe or poor batches of eggs can often be improved by letting the eggs stand for hours in sea water. This was generally true of the eggs used in these studies. Periodate treatment facilitated excellent fertilization of fresh eggs, but it was necessary to accept 5 to 6 per cent oocytes. Thus, the improvement in fertilizability on aging is doubtless related to the fact that continued protein synthesis under these conditions supplements the yolk stores and depletes the non-protein amino acids to the low level which appears to be characteristic of the fully ripe egg. In view of these findings, the early post-fertilization changes in non-protein amino acids (and probably some other metabolites) must apparently be regarded as adjustments which bring an initially flexible system to a more rigidly defined state from which normal development can proceed. At the same time it should be kept in mind that there is also a certain metabolic flexibility in the embryonic development of the sea urchin which appears to be correlated with the history of the eggs used (cf. 25).

The sharp increase in proteins between 20 and 27 hours of aging may be connected with the loss of fertilizability, which occurred in this interval. The jelly coat may also be of importance. The jelly dissolves slowly from the eggs, especially if they are agitated, and in any case it is usually lost when the eggs are centrifuged. However, the loss of fertilizability of aged eggs was observed to be often accompanied by a precipitation of the jelly. Since the precipitated jelly may be retained on centrifugation, it might account for the later protein increase, particularly when we take into consideration the fact that the drop in non-protein amino acids in the same period is not so marked. A more detailed study of the aging process, perhaps on jelly-free eggs, and with samples taken at more frequent intervals appears to be desirable.

It has already been noted that the soluble proteins of the egg may consist entirely of yolk proteins, whereas the insoluble proteins include, besides yolk, nucleoproteins and probably most of the structural and other non-yolk proteins. If this is the case, and it is principally yolk-protein which is synthesized when unfertilized eggs age, the change in composition of the insoluble proteins could be expected to be greater than that of the soluble proteins. Although the changes in the composition are small, it is found that the average change of the percentages of the amino acids after 7 hours of aging is 37 per cent greater in the insoluble protein fraction (3.38 as compared to 2.27). On the other hand, if the soluble proteins in the 50-hour pluteus are chiefly new proteins, by the same line of reasoning the change in their composition to this stage could be expected to be greater than that of the insoluble proteins. This is also found to be the case (12.3 as compared to 6.78).

DISCUSSION

The pattern of metabolic changes which occur in embryonic sea urchin development is apparently more complex than suspected from earlier studies. Because of this complexity and the relative rapidity with which metabolic changes take place, it will be highly desirable in future studies to collect samples at as frequent intervals as possible, particularly after the mid-blastula stage. It is known from studies with *Strongylocentrotus p.* (25) that the initial decline in free amino acids after fertilization is not a characteristic feature of development. This decline apparently takes place after fertilization of eggs which are removed from the ovary before they are fully ripe, when the concentration of non-protein amino acids is high. Under these conditions the non-protein amino acids may be a preferred energy source, whereas in ripe eggs, in which their concentration is low, the energy may come entirely from other sources. The initial decline in the proteins is probably a characteristic feature of early development, for the author has observed it in both species studied, and Örström (40) found a 3 per cent decline in protein nitrogen of *Paracentrotus l.* 10 minutes after fertilization. Although there is a net drop in protein amino acids to stage 3, a synthesis of soluble proteins begins at about stage 2 and continues to stage 4 (Figs. 1, 2, 4, 5). There may also be a synthesis of new insoluble proteins (as suggested by a finding of Hultin's (23)) which is not detected because it overlaps with the sharp decline in yolk proteins. Oxidative degradation probably plays a major role in this net loss of amino acids to stage 3. After stage 3 the changes in protein and non-protein amino acids are more or less complementary.

There are 3 waves of intense protein synthesis, one in the mid-blastula, another after hatching is completed and continuing into the late mesenchyme blastula, and a third in mid-gastrulation. A gradual protein synthesis occurs in early cleavage (in the soluble fraction), in the prism as the skeleton forms, and during the formation of the pluteus. There are 4 periods of intense yolk-protein breakdown, one during early cleavage, a second in the late blastula and extending through hatching, a third just before gastrulation and the fourth in the late prism and very early pluteus. The breakdown of soluble yolk in early cleavage appears to be slight. In the late prism, when the greatest wave of insoluble protein breakdown begins, there is no loss of soluble protein, probably because all the soluble protein reserves have already been consumed. It is possible that one or more waves of insoluble protein breakdown and synthesis occur after stage 20 but have not been detected because of the lack of closely spaced determinations after 28 hours.

A number of investigations have been carried out on the respiration of sea urchin oocytes, under-ripe, ripe and over-ripe eggs, and early embryos (cf. 34, 5, 6) of which the most recent and complete are those of Borei (5, 6). The results of the present investigation cast a new light on the interpretation of the respiratory data. As a starting point, let us consider the finding (5, 6) that when the egg of *Psammechinus miliaris* is removed from the ovary its respiration is relatively high but declines rapidly, reaching a low and constant value after 6 or 7 hours. Considering the data presented here, the high initial respiration of the egg is probably due in large part to the high oxidative energy requirement for the synthesis of yolk proteins. In this connection

Mestscherskaya (cf. 7) has shown that the respiration of the frog oocyte is greatest at the time of intense yolk synthesis. The respiratory decline to a low and constant value after 6 or 7 hours is probably due to the gradual relaxing of the oxidative energy demand as yolk-protein synthesis also comes to a halt. The low constant respiration is probably simply the residual respiration corresponding to the oxidative energy requirement for the maintenance metabolism of the resting cell. In connection with the sharp protein increase between 20 and 27 hours it is interesting to note that a rise in the respiration of the aged unfertilized egg also occurs at the time when fertilizability is lost (43, 49).

The determinations of the oxygen consumption of *Psammechinus m.* oocytes and under- and over-ripe eggs (230 minutes after removal from the ovary) showed that the respiration of the oocyte exceeds that of the under-ripe egg, that of the ripe egg ("ripe" = fertilizable) was still less, while the over-ripe egg consumed the least oxygen (5). Earlier studies (34) showed that the *Paracentrotus l.* oocyte has a greater oxygen consumption than the mature egg. These results are all in excellent agreement with the concepts outlined above according to which yolk-protein synthesis would be expected to be most intense in the oocyte and decrease in intensity as the egg ripens.

It has been found that the oxygen consumption of the fertilized eggs of many species varies within relatively narrow limits, whereas for unfertilized eggs the values are widely scattered (50). In view of the findings of Tyler and Humason (49), Borei (5, 6) and those presented here, the radically different respiratory changes attending fertilization are more readily comprehended. It is to be presumed that the respiration of unfertilized eggs will be more uniform if the measurements are performed after a certain period of aging when the oxidative energy requirements are for maintenance metabolism alone. This entire problem could probably be further elucidated by studies of the amino acid metabolism in the aging eggs of many species.

The changes in the respiration of the developing sea urchin embryo have been studied by Gray (cf. 5), Lindahl (33), and Borei (5). They have found the respiration to be monotonically increasing in development, with essentially the same pattern of changes having been detected by all three investigators. In its broadest aspect this monotonic respiratory increase doubtless reflects the gradual increase in oxidative energy requirements for maintenance metabolism and embryonic activity (swimming, rotation, etc.) as the relatively inert storage substances of the egg are converted to the metabolically active substances of the embryo. Superimposed upon this monotonically increasing requirement will be a varying demand for the work of the morphogenetic processes themselves, to which the sharply increasing phases of the respiratory curve are probably to be attributed.

At the time of fertilization of the sea urchin egg an abrupt change in the amino acid metabolism takes place. Not only does the synthesis of yolk cease, but a period of intense yolk-protein degradation ensues (Figs. 1, 2, 4, 5) and continues to about the 16-cell stage. This yolk-protein degradation appears to be a characteristic feature of early cleavage and it is probably a major source of energy. In early cleavage stages the R.Q. is consistent with protein or fat degradation (for references see (39, 7)). In addition, Hutchens *et al.* (24) have found that little or no carbohydrate is consumed at this time (depending upon the batch) but that considerable oxidative degradation of proteins appears to take place (judging from NH_3 production). The relatively low respiration of the embryo during early cleavage stages (cf. 5) is probably a reflection of the comparatively low oxidative energy requirement (after stage 3 protein degradation is primarily hydrolytic). These changes in amino acid metabolism provide further evidence that different substrates are utilized before and after fertilization, and that it is the decreasing component of respiration of the unfertilized egg which is eliminated by fertilization (cf. 6).

The second phase of the respiratory curve (see especially the well-documented curve of Borei (5)) consists of a steep respiratory increase beginning at about the 64-cell stage and terminating at about the time of ciliation some 2 or 3 hours before hatching. This is exactly the period when the first major wave of protein synthesis takes place, and the sharp respiratory increase undoubtedly reflects an increased oxidative energy requirement for this process. Extending an hour or so before and after hatching begins, is a more or less level respiratory phase which corresponds closely to the second major wave of yolk-protein breakdown. It seems likely that the oxidative energy requirements do not increase during this phase because hydrolytic protein degradation becomes a major source of energy. About 2 hours after hatching begins and an hour or so before the migration of primary mesenchyme, a second steep respiratory rise sets in. This is the time when the greatest wave of protein synthesis starts. This correlation, like the previous one, probably also hinges upon an increased oxidative energy requirement for protein synthesis. The respiratory determinations have not been carried beyond the early part of the second increasing phase, except for 2 or 3 much later determinations (33) upon the basis of which it is not possible to draw conclusions about the fine structure of the curve.

Thus, there are strong indications that developmental respiratory changes are directly influenced by and reflect changes in the energy metabolism of the embryo occasioned by the energy demands for protein synthesis and the energy supply from yolk-protein degradation. From this correlation of the respiratory changes with the amino acid metabolism, it appears that the energy for protein synthesis is derived from the concomitant oxidation of non-protein substrates. In this regard, the first wave of protein synthesis corresponds to the respiratory rise which is inhibited by Li^+ and which Lindahl believes to correspond to the oxidation of carbohydrates (33). The energy derived from hydrolytic yolk-protein breakdown does not seem to be stored to any appreciable extent for use in subsequent protein synthesis, or if it is, the energy required for synthesis greatly exceeds that available from the preceding wave of yolk-protein hydrolysis.

Since the respiratory curve increases monotonically, the oxidative energy requirements for maintenance metabolism and embryonic activity apparently keep pace with those for the synthesis of new embryonic proteins through the periods of intense protein synthesis, so that the high level attained is maintained after the requirements for synthesis slacken. In other words, the oxidative energy required for the maintenance of the new protoplasm and for embryonic activity is as great and perhaps greater than that required for the developmental processes themselves. For this reason, the curve for total new protein synthesis (Fig. 4) is very similar to the corresponding part of the respiratory curve, and it probably approximates the developmental increase in energy requirements for maintenance and activity.

Similar respiratory changes have been found to occur in a wide variety of embryos (cf. 48). These changes can, moreover, be correlated with characteristic changes in the yolk (48). Thus, there is some reason to believe that the alternate waves of yolk-protein degradation and synthesis of new proteins, which are found in the sea urchin embryo, may be of general occurrence. It is of interest to note in this connection, that the second respiratory phase of the embryos of some species (cf. (48) for references) is not level but declines somewhat. This may merely mean that the oxidative energy requirement for the synthesis of new proteins results in a respiratory increment greater than that of the requirement for maintenance and activity after completion of that synthesis. Whether this occurs could depend as much on the oxidative energy requirements of an intense protein synthesis as on the amount of energy supplied by a subsequent intense yolk-protein hydrolysis.

A study of the total individual amino acid content of *Paracentrotus l.* embryos has been carried out by Gustafson and Hjelte (15). Their curves for the developmental changes of amino acids in total hydrolysates are to be compared to the curves in Fig. 3 (total amino acid). In general it can be said that in the first 8 hours of development, in which period a valid comparison can be made, their curves do not show the uniformity found in these studies, nor is the same general pattern of changes evident. For a number of amino acids, one of which is glycine, they find the concentrations at one time or another in development to exceed the values in the unfertilized egg, which is never the case in the present studies. The relative molar values of total amino acids found by them are in fairly good agreement with those found here (Table I). A closer comparison with their results will not be undertaken because of the following differences in experimental procedures and program: their studies were confined primarily to the total amino acids, the changes in which are relatively insignificant and unrevealing; they have collected and base their curves on only 3 stages (morphologically unidentified) after 8 to 9 hours of development, whereas it is in this latter period that the main metabolic changes in amino acids take place; their material consisted of a mixture of several batches of embryos cultured at different times (14); their results are expressed relative to nitrogen determinations, where the total nitrogen is assumed to be constant during the first 30 hours of development (as opposed to having each stage represented by the same number of embryos).

From a comparison of the present results with those of the author's earlier study, there would appear to be a number of differences between *Paracentrotus l.* and *Strongylocentrotus p.* The ratio of the egg volume of the former species (442,000 μ^3 (19)) to that of the latter (280,000 μ^3 (25)) is 1.58. The average of the ratios of the protein amino acids to one another is somewhat higher at 1.94, while the total glycine is in the ratio 3.12. On the other hand, the egg volumes from different animals and in different seasons can vary by as much as 50 per cent (cf., for example, (20)), so that the disparity between these ratios may not be significant. Although there is relatively more free glycine in *Paracentrotus l.*, the quantity of peptide glycine appears to be much less. The fraction of the total amino acid existing in the free form seems to be much lower in *Strongylocentrotus p.* The developmental changes in the free amino acids of *Paracentrotus l.* conform to the same general qualitative pattern, whereas lysine and, under certain conditions, glutamic acid and arginine showed atypical changes in *Strongylocentrotus p.* There was but little indication of cyclic changes in the protein amino acids in the earlier study (protein cysteine, methionine and tyrosine did give some indication of cyclic changes) and the cyclic changes in the non-protein amino acids were more or less confined to the period before gastrulation. In both studies an initial decline in the free amino acids was followed by a sharp rise during early cleavage, but the subsequent decline occurs earlier in *Paracentrotus l.* Most of the amino acids appear to be present in peptides in readily detectible amounts in *Strongylocentrotus p.*, but the pattern of developmental changes is not entirely synchronous with that of the free amino acids.

These differences are believed to be partly the result of differences in experimental procedures. Runnström (45) has found that when eggs are fertilized in the presence of sodium periodate, fertilization, membrane elevation and the subsequent development of the embryo are often greatly improved. It is probable that the metabolism of individual embryos in a large batch obtained from many animals gets more and more out of step as development proceeds. Periodate may affect some system in the embryos in such a way that all obtain the same uniform 'start' upon fertilization, and develop in greater synchrony than would otherwise be the case. The *Strongylocentrotus p.* eggs were not fertilized in the presence of periodate so that developmental synchrony may have been poorer. Since the cyclic changes are abrupt and of short duration, overlapping in a large number of eggs would tend to lead to cancellation. It is also possible that species or batch specific variations in the pattern of correlation of protein synthesis and degradation (see p. 537) play a large role in determining the net changes in the amino acid fractions.

The finding of a relatively large 'peptide fraction' in *Strongylocentrotus p.* must be viewed in the light of the author's subsequent finding that small amounts of sea urchin proteins are soluble in 50 per cent alcohol. The changes in the 'peptide fraction' of *Strongylocentrotus p.* are thus probably influenced by the changes in the alcohol soluble proteins.

The failure to detect the full spectrum of cyclic changes in the amino acid fractions of *Strongylocentrotus p.* and the fact that only relatively small amounts of free amino acids were found, may also be related to differences in the extraction techniques. Alcoholic extraction of sea urchin embryos does not quantitatively remove free amino acids, for after extraction in a large volume of 50 per cent ethanol it is still possible to extract appreciable amounts of free amino acids with water (25).

The total molar content of amino acids (excluding free glycine) shows certain fluctuations in development and increases 7.7 per cent on aging for 7 hours (Fig. 1). The increase amounts to 67 per cent of the non-protein amino acids (excluding free glycine) in the unfertilized egg. The changes in non-protein amino acids do not exactly compensate for those in the protein amino acids but may be of either lesser or greater magnitude. Unless the embryos and unfertilized eggs are able to synthesize amino acids *de novo*, there would appear to be some evidence for the existence of an undetected pool of amino acid skeletons or derivatives which are readily convertible to the corresponding amino acids by the sea urchin embryo, but which are not converted to amino acids by hydrolysis and cannot substitute for the amino acid requirements of the assay bacteria. There was also some reason to suspect the existence of an undetected pool from studies of *Strongylocentrotus p.* (25). Such a pool might represent an intermediate form through which amino acids pass in the process of being incorporated into the sea urchin proteins. In this connection, Commoner *et al.* (11) have found that the amino acid residues of tobacco mosaic virus are not derived directly from the intact free amino acids of the host's pool, for the bulk of the nitrogen is derived from free NH_3, while Hultin (23) has shown that the nitrogen of labeled alanine when incorporated into the sea urchin proteins becomes distributed throughout the amino acids.

Although the discrepancies in the amino acid totals also occur when individual amino acids are considered (cf. Fig. 3), it should be kept in mind that there are certain hydrolytic losses due both to destruction and racemization. Furthermore, the conditions of hydrolysis vary, especially with respect to the ratio of moles of protein or peptide amino acids in the different fractions and different stages to the amount of acid used for hydrolysis. In the free amino acid assays, in particular, there is some stimulation extraneous to that under assay which is at present unavoidable, while some utilization of hydroxy and keto analogues can occur (22). These factors might possibly account for some of the periods of apparent increase in total amino acids but it does not seem likely that they could account for the increase on aging.

With the use of the techniques of this study it is possible to pinpoint periods of embryonic sea urchin development in which protein synthesis or degradation predominate. Since metabolites other than amino acids are accessible to micro-assay determinations, a new vista of quantitative correlative biochemical studies is in sight. Correlative studies of vitamins and other growth factors have been initiated (28) using the same extracts as for the non-protein amino acid assays of this investigation. The initial results of these studies show that a close correlation exists between the metabolism of amino acids and of vitamins (as judged from quantitative changes in vitamins and their conjugates (3, 27)). The changes are such as to permit further deductions about the amino acid metabolism, and they provide a new and unique sort of approach to the problem of vitamin function and the mechanism of cellular or embryonic control over metabolic processes.

These studies are being extended to other batches of normal, animalized and vegetalized embryos.

SUMMARY

The free amino acids and the amino acids of peptides and soluble and insoluble proteins have been quantitatively determined by microbiological assay of extracts of 22 stages of early development and 4 'stages' of aged unfertilized eggs of the sea urchin *Paracentrotus lividus*. In any given fraction, the 17 amino acids assayed show a remarkably uniform developmental pattern, although large concentration changes take place and the patterns of changes are different for the different fractions. The changes are generally abrupt, of short duration, synchronous, and either complementary or supplementary to one another. These changes appear to be caused by the degradation of reserve yolk proteins to free amino acids and peptides and the synthesis of new embryonic proteins therefrom. The developmental changes in the peptide (or bound) amino acids are very similar to those of the free amino acids.

There are at least 3 major and 3 minor periods of new protein synthesis and 4 periods of intense yolk-protein breakdown. Yolk-protein degradation and the synthesis of new embryonic proteins take place at more or less different times, so that the changes in the protein and non-protein amino acids occur in complementary cyclic waves.

Protein synthesis takes place in unfertilized eggs which are allowed to stand in sea water, and the non-protein amino acids are depleted to a relatively low level. This is probably a continuation of the process of yolk-protein formation which occurs as the egg ripens *in situ*.

The soluble and insoluble proteins show a different pattern of developmental changes, although the major periods of protein synthesis and degradation usually occur simultaneously in both. Analysis of these changes indicates that the soluble proteins in the unfertilized egg are almost entirely yolk proteins, which are consumed before the early prism stage. The insoluble proteins appear to consist mainly of yolk proteins but also include the structural and other functional components of the egg.

The periods of yolk-protein breakdown and new protein synthesis show a close correlation with known respiratory changes, and a new interpretation of these changes is given. Periods of intense protein synthesis correspond

to those of marked respiratory increase, while intense yolk-protein breakdown takes place at times when the respiration is known to remain more or less constant. It appears from these correlations that the energy for protein synthesis is derived primarily from concomitant oxidations, and that the energy from the hydrolytic degradation of yolk proteins is not stored to any great degree for use in subsequent protein synthesis but is consumed at the time it is generated.

There is some evidence for the existence of an undetected pool of amino acid skeletons or derivatives, possibly an intermediate form through which amino acids pass in the process of protein synthesis.

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THE FORMATION FROM THE SCHWANN CELL SURFACE OF MYELIN IN THE PERIPHERAL NERVES OF CHICK EMBRYOS[1]

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In 1953 the presence of the characteristic concentric lamellar structure of the myelin sheath was demonstrated in sciatic nerves of chick embryos (2). In the fibers from embryos incubated for 18 days, the alternate dense and less dense bands of the myelin sheath were closely packed, with a unit of spacing of about 97 Å. There were about 24 layers (a layer being one dense band plus the less dense region separating it from the next dense band) around each fiber. It was also reported that in preliminary studies of earlier stages (14 and 15 days of incubation) many fibers with as few as 2 to 12 layers were observed. The authors concluded that the increase in myelination of peripheral nerve fibers in the embryo proceeds by the addition, in sequence, of layers of oriented lipid and protein to the surface of the axon within the cytoplasm of the Schwann cell, rather than by accumulation and aggregation of droplets of lipid-protein with sudden "crystallizing out" of the characteristic fine structure. In this paper, evidence is presented which shows that the layers of oriented lipid and protein are derived from the Schwann cell surface by an infolding process initiated at the time the Schwann cell envelops the outgrowing axon.

EXPERIMENTAL RESULTS

The methods used in these experiments were the same as described in the previous paper (2) except that in some cases, the fixative (osmium textroxide) was dissolved in Locke's solution. All sections are cross sections through sciatic nerves.

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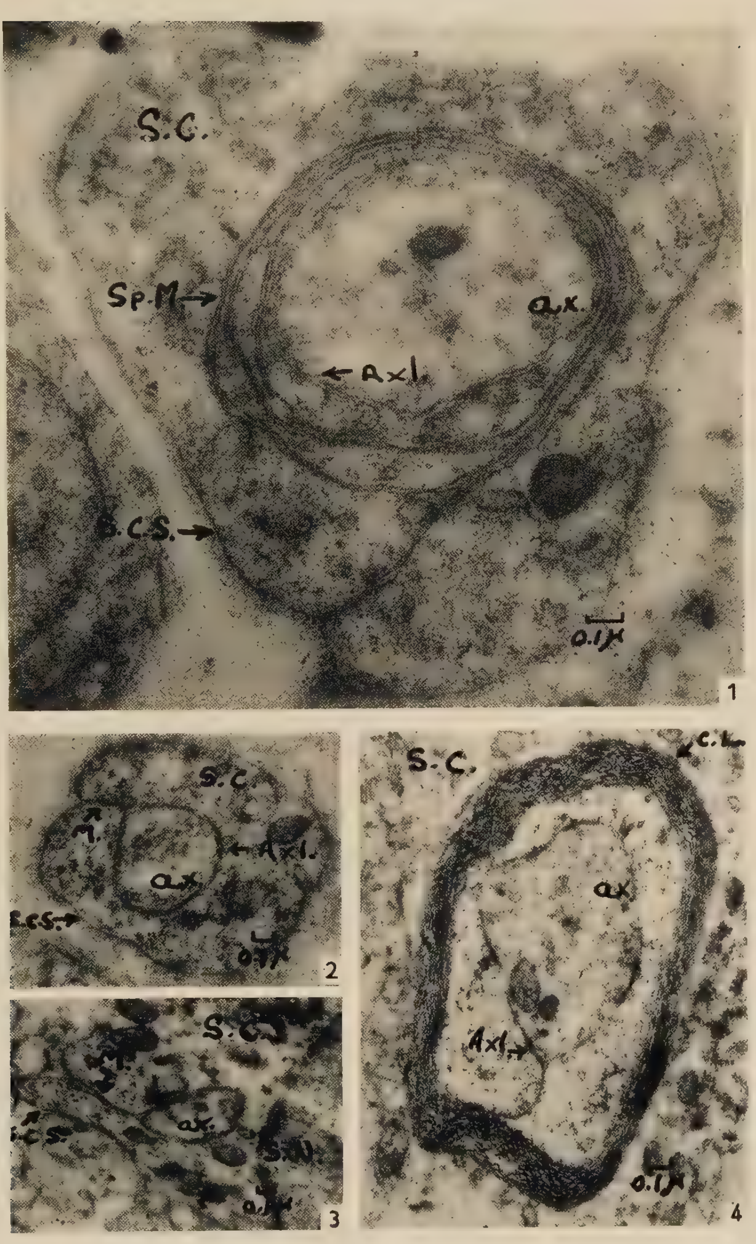
S.C.
Sp.M.
ax.
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1
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2
S.C.
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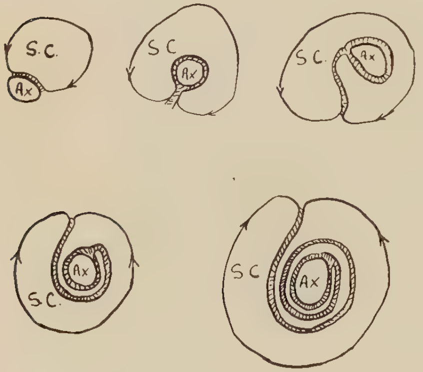
S.C.S.
ax.
S.N.
0.1μ
3
S.C.
C.L.
ax.
Axl.
0.1μ
4

Figs. 1–4. Cross sections through sciatic nerves of chick embryos. Fixation: 1 per cent osmium tetroxide in Hank's balanced salt solution or Locke's solution, about pH 7.4.
ax.—axon; *axl.*—axolemma; *M.*—membrane; *Sp. M.*—spiraled membrane; *S.C.*—Schwann cytoplasm; *S.C.S.*—Schwann cell surface; *S.N.*—Schwann nucleus; and *C.L.*—Compact layering.

The following general morphologic features have been consistently observed. The number of layer in the myelin sheath is smaller, the younger the embryo. The stage of development of the nerve fibers is not a function of the number of days of incubation of the egg, but is correlated with the size and development of the whole embryo. Myelination does not begin at the same time in all fibers of a single embryo; thus there may be some fibers with only two to four layers when a majority of fibers have 18–24 layers, and a few fibers with 10 or 12 layers when most fibers have 2–4 layers.



5

The layers of the myelin sheath in the early stages of myelination are observed as dense-edged "membranes" separated from each other by wide (compared to the thickness of the membranes) spaces in which there is Schwann cytoplasm and its inclusions (Fig. 1).

The first stage of myelination is the envelopment of the nerve axon by the Schwann cell (Fig. 2). The electron dense layer at the periphery of the axon is surrounded by the electron dense layer at the inner edge of the Schwann cell forming thus a double edged "membrane" (hereafter, the axolemma) (Fig. 2). The outer dense edge of the axolemma is not closed but retains its attachment with the Schwann cell surface from which it is derived by continued infolding of the latter to form a double edged membrane (Fig. 3).

At the next stage, this membrane which connects the surface of the Schwann cell with the axolemma is observed as a spiral (Fig. 1).

Fig. 5 is a diagrammatic presentation of these early stages of myelin formation.

DISCUSSION

The spiral appearance of the early myelin membrane in cross section could reflect either a pattern of growth of the Schwann cell surface about a stationary axon or a revolving of the axon within the Schwann cell. The derivation of the myelin sheath membranes from the Schwann cell surface indicates a similarity of molecular composition and arrangement.[1] The great increase in the infolded surface of the Schwann cell during myelination suggests the incorporation into the myelin membrane of lipid and protein in large amounts.

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[1] Polarization optical studies of the myelin sheath have shown the radial orientation of lipid molecules and the tangential orientation of protein molecules (6, 7). There is no conclusive

The unit structure in the early stages of myelination is a dense edged membrane which varies greatly in thickness (100–300 Å as measured from the outer edges of the limiting dense bands). Later in embryonic development the myelin shows compact layering (Fig. 4) of which the structural unit is a layer of uniform thickness (about 100 Å) consisting of one dense band and one less dense band. The mode of rearrangement of the double edged membrane into the compact layering of the fully developed myelin sheath is not understood. It is conceivable that outward pressure from the radial growth of the axon could facilitate such a rearrangement. Alternatively, a variety of intracellular movements might bring successive layers of the spiraled membrane into apposition sufficient for specific molecular interactions to provide the main force for "zippering" them together. Such a process proceeding from the inner part of the spiral to the outer would allow for the expulsion of Schwann cytoplasm and its particulates from between the layers of the membrane. Knowledge of the molecular structure in the dense and less dense bands of both the spiraled membrane and the compact layers may be furthered by experimental work on the interactions of osmic acid with purified lipids and lipid-proteins (3). Such experimental work is in progress.

The structure of the axolemma differs from that of the other layers of the spiraled membrane of the early myelin in that both the original axonal surface and the Schwann cell surface contribute to the formation of this dense-edged membrane. Fig. 4 illustrates the fact that this membrane may retain its integrity as a unit when the axon is detached from the myelin by shrinkage during fixation, dehydration or embedding. The axolemma and the extension of its outer dense layer as a membrane to the surface of the Schwann cell as shown in Figs. 2 and 3 is analogous to the form of unmyelinated ("C") fibers described by Gasser (1). The attachment of the fiber to the surface of the Schwann cell was called a "mesaxon" by Gasser. The axolemma is also analogous to the axon-Schwann cell interface described in intervertebrate metatropic fibers by Geren and Schmitt (4).

A more detailed paper will be published later. The data presented here suggest that the lipid-protein membranes of cell surfaces may fuse to form

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polarized light evidence of similar molecular arrangement in the plasma membrane of cells other than red cells although there is indirect evidence that both lipids and proteins are major constituents (5).

intracytoplasmic, lipid-protein containing structures. The mode of formation from Schwann cells of mitochondria-like particulates in the axons of invertebrate nerve fibers as suggested by Geren and Schmitt (4) would be another example of this general phenomenon.

The technical assistance of Miss Phyllis Hoffman is gratefully acknowledged. Miss Josephine Raskind collaborated in the early phases of the experimental work. The author would like to express appreciation to Dr. F. O. Schmitt and to Dr. Herbert Gasser for their interest in and criticism of this work.

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Experimental Cell Research, **7**, *563–564* (*1954*)

BRIEF NOTES

EFFECT OF COLCHICINE ON SPERMATOGENESIS IN CERTAIN GRASSHOPPERS

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Received June 17, 1954

Levan (6, 7) and Barber (1) have reported profound changes in the pairing of chromosomes and formation of chiasmata following treatment with colchicine in certain species of *Allium* and *Fritillaria*, respectively. In connection with an investigation concerning the mechanism of chiasma localisation, the present writer has studied the action of colchicine on the spermatogenesis of the grasshoppers *Mecostethus grossus*, *Stauroderus bicolor*, and *Chorthippus elegans*. The first of these is the classical example of terminal procentric localisation of chiasmata (5, 10, 12), while the others do not show any obvious determination of the position of chiasmata.

About 0.1 to 0.05 mg of colchicine (a 0.1 per cent solution in 0.6 per cent saline) per 100 mg of body weight was injected into the abdomen of the experimental animals. These were killed after intervals of 3, 6, and 12 hours and of 1 to 4 days. The colchicine clearly impaired the viability of the insects, apparently owing to a decreased intake of food. No change in the oxygen consumption of the animals was detected with the Barcroft differential type of respirometer. The cytological action of colchicine was clearly manifested as a complete arrest of the gonial and spermatocyte divisions during the experimental period.

The present results suggest that the insects may be unable to eliminate the colchicine completely once it has been introduced into the tissues. In spite of this, no conspicuous action is exerted on the early phases of meiosis. Zygotene and pachytene look quite normal, and the pattern of chiasma formation remains unchanged in all the three species investigated. Owing to the arrest of mitosis no new spermatocytes can be formed as long as the action of colchicine prevails. The increase in the number of blocked first metaphases shows, however, that the course of meiosis is not brought to a standstill.

The effect on the spermatogonial metaphase chromosomes is, as a rule, a shortening and thickening of the chromosome body, evidently as a consequence of overspiralisation (cf. 3, 4, 9). The first metaphases, distinguished from the diakineses on the basis of the disappearance of the nuclear membrane, are of two different types. One resembles the usual *c*-mitosis with the bivalents overspiralised and often gathered together in the middle of the cell. The other type resembles diakinesis with the bivalents widely scattered. The chromosomes are relatively long and slender, but the bivalents have opened out completely with strictly symmetrical chiasmata. At present, it is not possible to state with certainty whether the second type is a later phase in development than the first, or vice versa. In any case, it is seen that colchicine cannot have a direct and absolute contracting effect on the chromosomes, as has been suggested by certain authors (8), but acts only by inhibiting in one way or another the factors which normally control the spiralisation cycle of the chromosomes.

The dual result of colchicine action is sometimes found in mitosis also. Thus, the contracted type of first metaphase described above may correspond to the "star" and "ball" metaphases, and the despiralised type to the "exploded" metaphase observed in the epithelial mitoses of the newt (2). The absence of "exploded" metaphases in mammalian tissues, for instance, after treatment with colchicine for 6 to 10 hours (11), favours the view that this type may be a later stage in the development of *c*-mitosis.

The ineffectiveness of colchicine on chromosome pairing and chiasma formation in the present material can hardly be due to the more prolonged treatment usually applied in plant experiments. It is possible that four days may be too short a time interval for the progress of a single cell from leptotene to diplotene. But an immediate action on the early phases of meiosis should still be revealed during this or even a shorter period, since some effects may be recognizable even earlier than diplotene, and cells at all stages of meiosis are present from the beginning of the experiment in the grasshopper testis. In contrast to this, meiosis is usually more or less synchronous in the whole flower bud of plants. In *Allium*, unpaired pachytene threads were seen just after the end of treatment, and a decreased chiasma frequency was observed at first metaphase on the 5th to 7th day after treatment (6). The differences found between the reactions of animal and plant materials are in accordance with the conclusion that the determination of pairing depends on one side on the polarisation of the chromosome ends in grasshoppers and most other animals, while on the other side it depends on the polarisation of the centromeres in such plants as show a pattern of chiasma formation similar to that in *Allium* and *Fritillaria* (10). Colchicine may inhibit the specific activity of the centromeres (1), but is evidently without effect on the polarisation mechanism of the chromosome ends. Observations on the action of colchicine on meiosis thus furnish experimental support for the view that chiasma localisation is brought about primarily by an orderly initiation of pairing in certain chromosome regions with a time-limit as an additional factor (10).

This study has been aided by a grant from the Leo and Regina Wainstein Foundation. A more detailed report will be published later.

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INFRARED ABSORPTION STUDIES OF FOWL AND MAMMALIAN ERYTHROCYTES[1]

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An imposing amount of evidence has been accumulated indicating that biological systems can be studied by infrared spectroscopy (1, 2, 5, 6, 12, 14), revealing in certain instances the applicability of the method for identification of the constituents of complex mixtures (8, 9, 12, 13). During an investigation dealing with the infrared spectrum of influenza virus which had been adsorbed onto chicken red blood cells (16), the spectra of chicken and human red cells were determined. It was observed that the spectra of chicken and human red cells were consistently different, thus initiating a study on the possibility of distinguishing erythrocytes from different animal species by infrared spectroscopy.

Human, guinea pig, sheep, dog, chicken, duck and pigeon blood specimens were collected in Alsevers solution and the cells were washed three times in saline. Approximately 0.2 ml of various concentrations of cell suspensions was spread on the surface of 1 square inch silver chloride plates and dried at 37° C. Differences in film thicknesses had no observable effect on the data reported here. The infrared absorption spectra from 2–16 μ were determined with a Baird-double beam recording instrument equipped with a sodium chloride prism.

The spectrum of human red cells was similar to that reported by Blout and Mellors (6) and Woernley (15). The usual absorption bands of proteins were seen; namely, N—H and O—H stretching at 3.0 μ, C—H stretching at 3.4 μ, C—O stretching at 6.05 μ, and C—N bending at 6.45 μ. The most informative region of the spectrum (6–12 μ), although complex in terms of interpretation, is shown in Fig. 1. The spectra of pigeon, duck and chicken erythrocytes were similar, but differed consistently from the spectra of guinea pig, sheep, dog, and human red cells. The fowl cells showed greater absorption than the mammalian cells at 8.1, 9.1, 9.3 and 10.4 μ; whereas a shallow band at 10.2 μ in the spectra of mammalian cells was not present in the spectra of fowl cells. Ratios between absorbances (O.D.) of the bands at 9.1 μ and the absorbances of the protein bands at 6.45 μ were determined to quantitate the observed differences. The results summarized in Table 1 show significantly higher ratios for the fowl cells than for mammalian cells, and the ratios of pigeon cells were found to be higher than the ratios of the other fowl cells studied.

Obviously, the nuclei in the fowl cells were responsible for the more intense absorption at the wave lengths given above. To substantiate this finding, hemolysates were prepared by lysing chicken red cells through repeated sequences of freezing and thawing, and nuclear fractions were recovered by centrifugation of the lysed chicken cells. The infrared spectra of these preparations, as shown in Fig. 1, would indicate that the substances responsible for the distinctive features of the fowl cells resided in greater concentration in the nuclear fraction (Table 1). The peak of the broad band at 9.1–9.3 μ was shifted to about 9.45 μ. The hemoglobin fraction yielded a spectrum with considerable attenuation at those regions that were found to be greatly intensified in the nuclear fraction. The nucleic acids were extracted from the nuclei according to a modification of Schneider's method (11), as given by Nowinski and Yushok (10). As seen in Fig. 2, the nucleic acid fraction (hot trichloracetic acid extract) yielded a spectrum similar to desoxyribonucleic acid (sperm), indicating that desoxyribonucleic acid is partly responsible for the absorption at about 8.1, 9.4, 9.8 and 10.3 μ. Neither the alcohol-soluble fraction nor the residue remaining after extraction presented spectra with absorption bands at all of these wave lengths. In this connection, other studies on the infrared spectra of desoxyribonucleic acid have demonstrated these same absorption maxima (3, 4, 8, 12, 15).

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[1] This study was supported in part by funds provided under Contract AF 18(600)-464 with the USAF School of Aviation Medicine, Randolph Field, Texas.

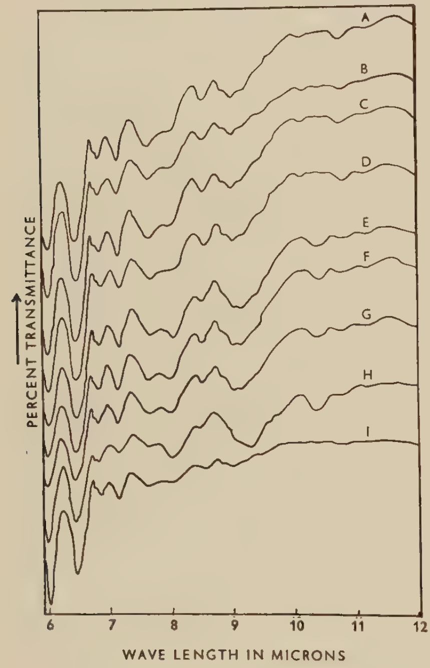

Fig. 1.

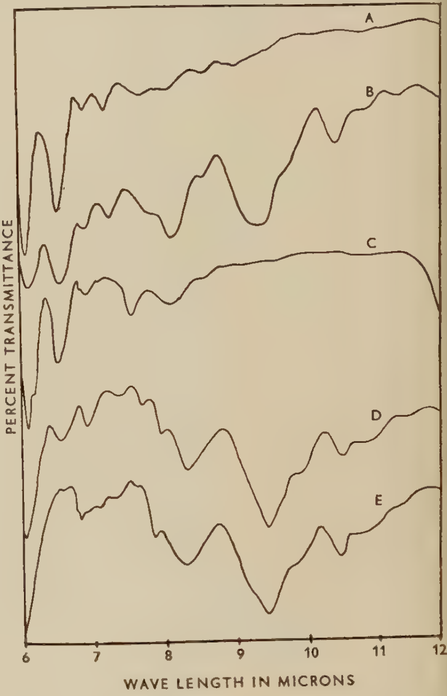

Fig. 2.

Fig. 1 (left). Infrared absorption spectra of fowl and mammalian red blood cells: (*a*) guinea pig RBC; (*b*) sheep RBC; (*c*) dog RBC; (*d*) human RBC; (*e*) pigeon RBC; (*f*) duck RBC; (*g*) chicken RBC; (*h*) chicken RBC nuclear fraction; (*i*) chicken RBC hemolysate.

Fig. 2 (right). Infrared absorption spectra of chicken RBC fractions and desoxyribonucleic acid; (*a*) hemolysate; (*b*) whole nuclear fraction; (*c*) alcohol-soluble fraction of nuclei; (*d*) nucleic acid fraction of nuclei; (*e*) desoxyribonucleic acid.

TABLE I

Relative estimations of substances responsible for infrared absorption at 9.1 μ in red blood cells.

Preparation	$\frac{\text{absorbance at } 9.1\ \mu}{\text{absorbance at } 6.45\ \mu}$
Guinea pig RBC	0.19
Sheep RBC	0.17
Dog RBC	0.16
Human RBC	0.16
Pigeon RBC	0.29
Duck RBC	0.26
Chicken RBC	0.24
Chicken RBC nuclear fraction	0.39
Chicken RBC hemolysate	0.11

It may be that substances other than the nucleic acids also contribute to the differences observed between the spectra of fowl and mammalian erythrocytes. Danielli (7) has emphasized the need for awareness relative to interpretation of tissue spectra. He noted that because of modification of a spectrum by interaction with neighboring molecules and masking of certain substances absorbing in the same vibrational region, careful interpretation of biologically complex spectra is required. It is felt that the infrared "finger-print" of the nuclear fraction of fowl cells reported here may be, in part, valuable to the understanding of the cytological distribution of molecular species.

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THE PREPARATION AND UTILIZATION OF WHOLE CELL SUSPENSIONS OBTAINED FROM SOLID MAMMALIAN TISSUES[1]

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This paper presents a method for preparing large quantities of suspensions of whole cells from such solid tissues as liver. Previous reports in the literature have described methods for obtaining relatively small quantities of whole cells (e.g. 3, 9, 13, 5, 1, 14). However, for a series of metabolic-morphologic studies on normal and neoplastic tissue, the earlier methods of preparing whole cells were unsatisfactory because of (*a*) low yields of cells, (*b*) the proportion of injured to uninjured cells was too large, or (*c*) the methods involved chemical agents, the effects of which on permeability and/or metabolism were unknown or doubtful.

The following method of preparing whole cell suspensions from solid tissue utilizes the simple expedient of screening the tissue through two or three (depending on the tissue) successively smaller mesh screens. The use of these cell suspensions for metabolic studies is illustrated by experiments designed to prove the integrity of the cells. A preliminary report was presented earlier (6).

Experimental. Suspensions of whole cells from rat liver were prepared in the following way. The animals were sacrificed by decapitation (this allowed some of the blood to drain from the liver), the liver was removed, immediately weighed, and placed in cold[3] isotonic salt solution.[4] The lobes of the liver were cut horizontally[5], and the cut surfaces placed on a 14–16 mesh stainless steel screen. The tissue was then forced through the screen by gently pressing with a washed rubber stopper. The screened tissue was scraped from the screen with a spatula, and placed in a small beaker containing about 5 ml of isotonic salt solution. The screened tissue was pooled with the unscreened tissue, i.e. the tissue which remained on the screen.

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[1] The major portion of this work was carried out while the author was a Brittingham Fellow in Cancer Research, Dept. of Chemosurgery and the McArdle Memorial Laboratory, University of Wisconsin, Madison, Wisconsin.

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[3] Greater activity and yield of cells was obtained when isotonic solutions were at approximately 10–15° C, rather than at 3–5° C.

[4] Isotonic saline or sucrose were used in oxidative experiments, Robinson's (12) bicarbonate-glucose medium was used in anaerobic experiments.

[5] The lobes were cut with either (*a*) a sharp scissors, or (*b*) a small brain knife while the liver was held between a glass slide and a piece of filter paper, i.e., similar to the Deutsch slice technique (2).

The resulting "brei" was stirred with a glass rod, in order to loosen cells adhering to clumps of tissue.

The "brei" was then screened through a 24 mesh screen and the screened material collected in a small beaker in a manner similar to that described above. However, in this step the unscreened material was discarded.

The resulting suspension of cells and clumps of cells was then screened through a 60 or 80 mesh screen. Very gentle pressure was used when forcing the cells through this screen. Although large clumps of cells are usually absent at this stage, they may be almost entirely eliminated by pouring the suspension through a 100 mesh screen.

The cells may now be washed by centrifuging in a horizontal head centrifuge.[1] A cell suspension almost free of blood cells was obtained by centrifuging 6–10 ml portions of the suspension in 15 ml centrifuge tubes at 30–50 times gravity for one and a half minutes, resuspending in fresh isotonic salt solution and centrifuging at approximately 30 × G for one minute, and finally resuspending and centrifuging at 30 × G for one half to one minute. The cell suspension was then made up to a volume that would insure approximately 40 mg dry weight of cells per ml. With experience, this can easily be estimated, since about the same yield of cells per gram of liver will usually be obtained. The yield of cells depends somewhat on the tissue and the skill and experience of the operator. However, 50–100 mg dry weight of liver cells may quite easily be harvested from a rat weighing approximately 150 grams.

Morphological evidence that suspensions prepared by this method are made up of whole cells includes: intact cell membrane, unchanged nuclear-cytoplasmic ratio, and mitochondria that do not appear swollen. Moreover, under conditions for anaerobic glycolysis (7), the cells maintained their morphological characteristics.[2]

Several methods were used to define the functional integrity of the cells in suspension biochemically. The first method used was an application of the "cytolysis quotient" (10, 11). The principle of this method is that in whole cell suspensions no increase in succinoxidase activity would be expected on the addition of cytochrome *c*. This method was applied to whole cells obtained by the screening procedure. Reaction mixtures were made as described in "Manometric Techniques" (15). The results indicated that suspensions of whole cells showed little or no change in activity upon the addition of cytochrome *c*, whereas homogenates of these cells showed definite dependence upon cytochrome *c* (Fig. 1).

A similar effect was obtained when succinate was made the limiting factor. Suspensions of whole cells were entirely independent of exogenous succinate, whereas the cell homogenates were dependent upon succinate for optimum activity.

Although this evidence is indicative of the functional integrity of the cells, it is by no means conclusive, since only a single reaction system was investigated. Moreover, this method is not suitable for routine determinations, because of the care and experience required in handling cells in order that endogenous materials are not lost.

More conclusive evidence of the functional integrity of whole cells has been demonstrated by the experiments of LePage (8), in which radioactive glycine was incorporated into the protein fraction of a whole cell suspension from mouse liver. This suspension showed a sevenfold greater uptake of radioactive glycine than did a cell homogenate. These results were confirmed (6), and experiments are in progress to correlate these data with morphological and staining characteristics of the cells (unpublished data).

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[1] Centrifuging, i.e. washing, the cells is optional. LePage (8) used two washings in experiments comparing the uptake of radioactive glycine by normal liver and ascites cells.

[2] Before and after each experiment, smears were made of the cells, fixed in osmium vapor for 1–2 minutes, and stained with fast green as described by Harman (4).

Conclusions. The technique of using several successive screenings, as described above, results in obtaining large yields of whole cells from solid tissue. The large yields may be attributed to the light pressure required to push the tissue through the smaller mesh screens, since much of the congestion due to stroma is eliminated during the first two screenings.

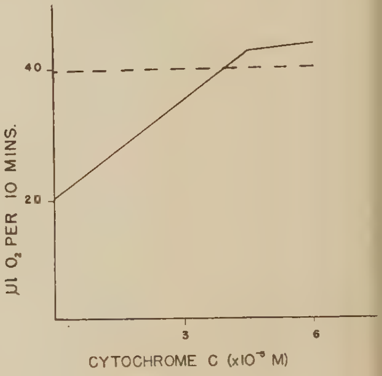

Fig. 1. Effect of cytochrome *c* on rat liver cell suspensions and homogenates. Cell suspensions represented by broken line, and homogenate of cells by solid line.

Although the use of this technique is being extended to include other organs and neoplastic tissues, and the preparation and maintenance of the cells is still being perfected, the results have indicated that the preparation and investigation of such cell suspensions is feasible and practical. Moreover, the experiments demonstrating the incorporation of radioactive glycine indicate that whole cells, prepared in this manner, function as intact units, and are superior to cell homogenates under similar conditions.

The advantages of a whole cell suspension over a cell homogenate are that the cell membrane and the intracellular structures are intact, and the intracellular constituents are not dispersed. The advantage over a tissue slice lies in the fact that permeability factors are greatly minimized, and the ratio of injured to uninjured cells is small. Furthermore, in a suspension of whole cells, each cell is in contact with added co-factors, substrated, hormones, etc., so that an immediate effect on the cell and/or metabolism may be observed; i.e., morphological and biochemical changes of the cell may be observed in a controlled environment. Moreover, suspensions of whole cells obtained from solid tissue permit metabolic and morphological investigations previously limited to bacteria and ascites cell tumors.

Suspensions of cells from solid tissues may also offer special advantages in fields other than metabolism and morphology, for instance the fixing of cells and examination of chromosomes (Scott, O. C. A., personal communication), ultraviolet and roentgen ray investigations of cellular constituents, and transplantation of solid tumors with known numbers of viable cells.

Summary. 1) A method is described whereby suspensions of whole cells, obtained in relatively large yields, were prepared from solid tissues.

2) The technique employs several screenings, using successively smaller screens.

3) The integrity of the cells was indicated morphologically by microscopic examination, and biochemically by (*a*) the independence of cell suspensions from exogenous cytochrome *c*, or succinate, in succinoxidase measurements, and (*b*) a sevenfold greater incorporation of radioactive glycine (anaerobically) into proteins of the cell suspensions over homogenized cell preparations.

4) Advantages and possible applications of whole cell suspensions are discussed.

The author wishes to express his appreciation to Dr. G. A. LePage for his helpful suggestions, and for extending to me the facilities of his laboratory.

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A NEW APPROACH TO FREEZE-DRYING OF TISSUE

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The techniques described in the literature for the freeze-drying of tissue all follow the same basic pattern. The tissue, after being rapidly frozen, is placed in a vacuum at – 30° C or lower, near a desiccant. The water is allowed to sublime off and when dry, the tissue is infiltrated with paraffin. With this general procedure animal tissue can be dried in 4 to 24 hours. Plant tissue is more difficult to dry, and with the same procedure requires 2 to 6 days.

The apparatus described here will freeze-dry plant tissue in 2–8 hours. The great acceleration in drying rate is accomplished by moving a stream of dry air over the tissue at – 30° C or lower, under a vacuum of 1.5 cm.

There are three main units to the system (Fig. 1). The first, from the point of view of entering air, is the air drier. This is a modified cold finger condenser, placed in a dry ice-methyl cellosolve mixture at – 75° C. The coil of tubing around the outside is to insure drying of all the air passing from the condenser. The stopcock on the inlet is used to regulate the flow of air into the system.

The second unit is the tissue dehydration chamber. This consists of a glass tube 3 inches in diameter and approximately 14 inches in length which has a fritted glass plate sealed in near the bottom and an industrial pipe flange top. The industrial flange consists of a thick glass collar with a groove in the upper surface in which is set a rubber ring and over that a solid brass disc. This is preferable to a ground glass joint because the latter frequently sticks. A coil of glass tubing approximately 6 feet in length is wound around the 3-inch tubing and sealed to the bottom. The function of the coil is to equilibrate the air to the temperature at which it is to pass over the tissue. The upper end of the tubing is attached to the outlet from the air drier. A large glass joint is built in near the top of the chamber as an outlet to the water trap and vacuum pump. The dehydration chamber is placed in a dry ice-methyl cellosolve bath at – 30° to – 40° C. The fritted glass plate insures equal distribution of the air before it reaches the tissue.

The third unit, attached to the tissue chamber by the large connection, is a conventional cold finger condenser that acts as a water trap. It is placed in the same dry ice-methyl cellosolve bath as the air dry unit. The function of this unit is to protect the vacuum pump which is attached to the outlet side of the condenser.

The tissue is held in metal containers with wire mesh bottoms placed in holes in a plastic disc. This disc sets directly on the fritted glass plate and has a plastic rod to act as a handle. To this rod is attached a thermometer which can be read through the wall of the chamber from the outside. The scale given on the diagram is not to be considered critical. Not included in the diagram is the flow meter and a column of chemical desiccant which is placed just before the air drier and a vacuum gauge which is connected behind the water trap.

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[1] Fellow, National Institutes of Health, National Cancer Institute.

The operation of this system is very simple. The temperature of the bath surrounding the condensers is lowered to − 75° C by adding an excess of dry ice to the methyl cellosolve. The temperature of the bath around the dehydration chamber is adjusted to − 30° to − 40° C by the careful addition of small amounts of dry ice to the methyl cellosolve. (The temperature of this bath will rise 1° to 2° C per hour during operation.) The tissue is frozen by dropping it into a metal container sitting in a bath of propane cooled by liquid nitrogen after the method of Moberger *et al.* (2). The metal container is then transferred to the dehydration chamber in the shortest time and distance possible. It is lowered into the hole in the plastic disc by long forceps or wire hook. When all the metal containers are in the holder the top of the dehydration chamber is set in place and the pump started. The pressure is regulated to 1.5 cm by adjusting the stopcock on the air drier. The temperature of the tissue dehydration chamber is regulated by adding small amounts of dry ice to the surrounding bath at 2 to 4 hour intervals. It has been found convenient to start a run in the early afternoon, watch the temperature until evening and then allow the apparatus to

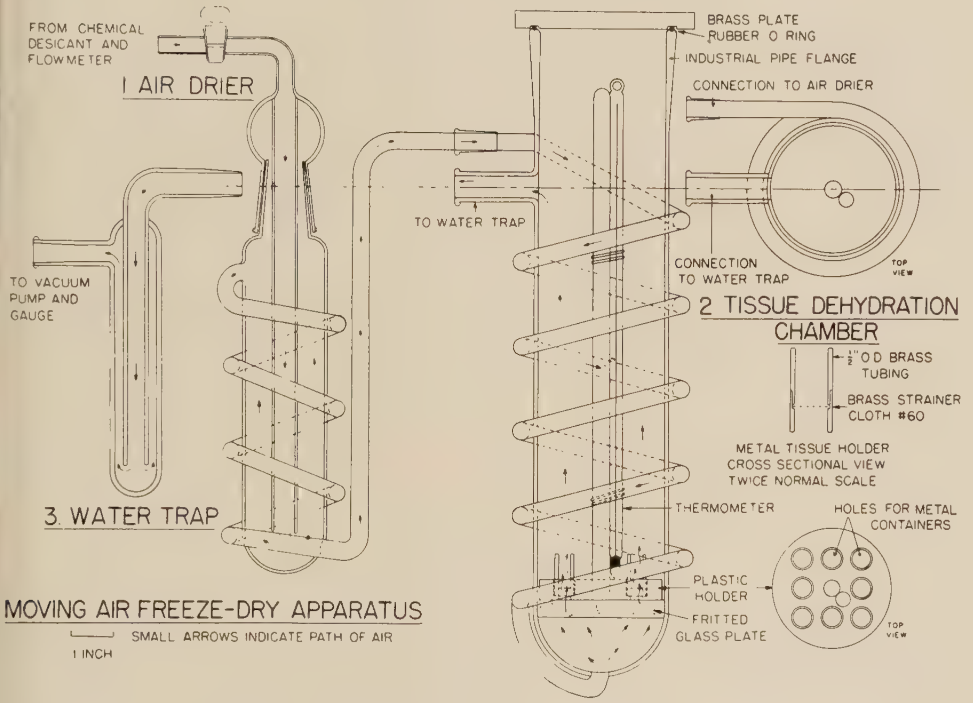
FROM CHEMICAL DESICANT AND FLOWMETER
1 AIR DRIER
TO VACUUM PUMP AND GAUGE
3. WATER TRAP
MOVING AIR FREEZE-DRY APPARATUS
SMALL ARROWS INDICATE PATH OF AIR
1 INCH
TO WATER TRAP
BRASS PLATE
RUBBER O RING
INDUSTRIAL PIPE FLANGE
CONNECTION TO AIR DRIER
CONNECTION TO WATER TRAP
TOP VIEW
2 TISSUE DEHYDRATION CHAMBER
½" O D BRASS TUBING
BRASS STRAINER CLOTH #60
METAL TISSUE HOLDER CROSS SECTIONAL VIEW TWICE NORMAL SCALE
THERMOMETER
HOLES FOR METAL CONTAINERS
PLASTIC HOLDER
FRITTED GLASS PLATE
TOP VIEW

Fig. 1. Diagram of the apparatus.

run unattended until the next morning. At the end of the run the tissue is removed and stored in a vacuum desiccator or infiltrated with paraffin in a second, higher vacuum.

The results of experiments determining water loss by change in weight indicate that with stem tissue, 80 to 90 per cent of the water is lost in the first two hours and that by four hours the tissue is oven-dry. With root tips eight to ten hours are required for oven dryness; with leaf tissue only two hours are necessary. Histologically the results are identical with those previously reported (1).

This apparatus has several advantages over the conventional methods. The most important is the shorter drying time. In addition, the apparatus is simple and inexpensive to make. Since a vacuum of only 1.5 cm is employed almost any vacuum pump can be used and the need for a diffusion pump is eliminated. A heater is not required to warm the tissue and a simple, low scale thermometer is adequate to measure the temperature. All the tissue is run at the same temperature and this temperature can be easily varied.

The author is especially grateful for the time and effort expended by Dr. Frank Salisbury during the development of the apparatus and to Professor Arthur Galston for the interest shown by him at all stages of the work.

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ADDENDUM

It has been brought to the attention of the author that this approach to freeze-drying is indeed not new, as Treffenberg (*Arkiv Zool.*, 4, 295 (1953)) has already published a similar method although employing a different design.

Experimental Cell Research, **7**, *575–577 (1954)*

THE DEMONSTRATION OF A MATRIX SUBSTANCE IN ISOLATED RAT LIVER MITOCHONDRIA[1]

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Received July 16, 1954

The conventional fixation of isolated mitochondria for electron microscopy consists of the treatment of the isolated material with fixatives (cf. 3) or fixation prior to the purification of the homogenates (8, 9) usually with employment of osmium tetroxide containing solutions. The methods have two main disadvantages: 1) Through extraction or denaturation, the treatment may cause changes in the morphology of the mitochondria as compared to the mitochondrion in the untreated suspension. 2) Further studies on the effect of various fixation and extraction procedures will be made impossible.

As in this Institute investigations are under progress which aim at the electron microscopical study of the composition of isolated mitochondria, an attempt was made to get a starting preparation, which eliminated the above-mentioned objections as well as giving good observational conditions for the two main constituents previously demonstrated in the mitochondria—the membranes and the mitochondrial inner bodies (3, 4, 5). The procedure employed is as follows:

Mitochondria were isolated by differential centrifugation as described previously (5). They were then suspended in double distilled water and washed three times, each time collected in a "Misco" high speed centrifuge (Microchemical Specialties Co.) run in a cold room at 0° C at 24,000 × g for 10 minutes. Drops of this suspension were placed on Formvar films covering electron microscopical screens, and left there in the cold for about 5 minutes. Then the drops were sucked off with a small glass capillary, and the still wet mitochondrial preparations allowed to dry at 0° C *in vacuo* at 10^{-3} mm Hg. After having attained room temperature *in vacuo* the screens were shadow-cast at an angle of 30° with palladium prior to observation in a Siegbahn-Schönander electron microscope at 50 kV.

The pictures obtained of the mitochondria are represented by Fig. 1. It is easy to identify the mitochondrial membranes (*A*) and the localization of the granular inner bodies (*B*). But, in addition, these preparations show a very finely granular substance surrounding the other structures (*C*). To elucidate the question about the origin of this finely granulated substance the effect of treatment of the preparations with different fixatives and solvents was investigated. In the course of these experiments, which will be reported fully later on (6), it could be shown that the substance disintegrated under the influence of distilled water. As the mitochondria

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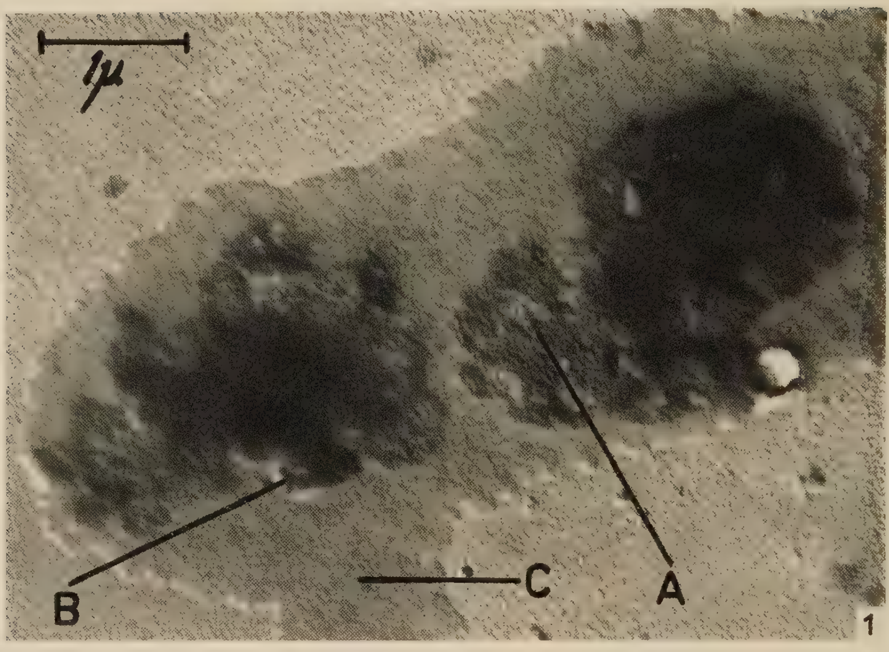

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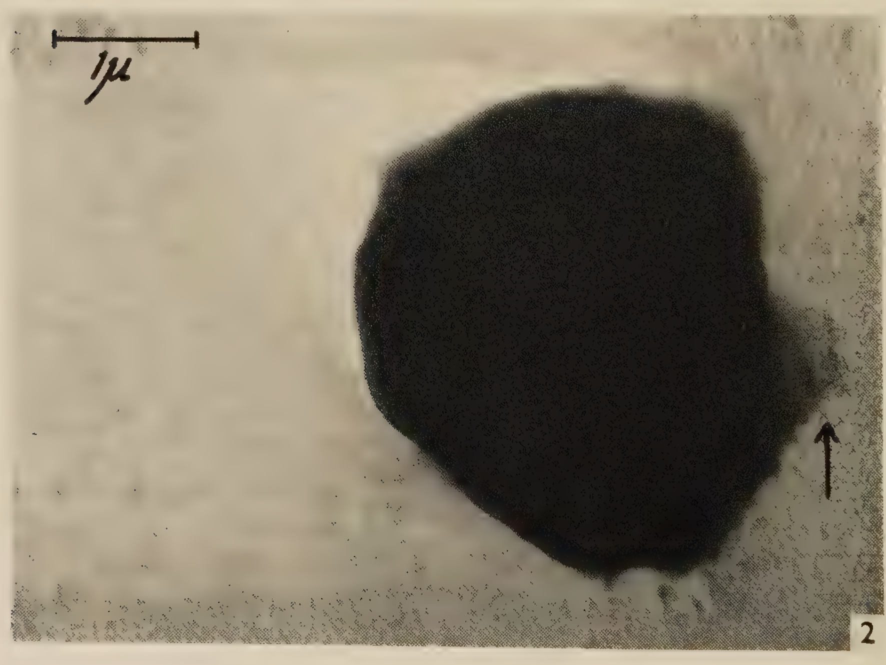

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were originally suspended in distilled water, it could be assumed that the pictures obtained might result from the disruption of the mitochondrial membranes caused by the drying in high vacuum of the spherical mitochondria swollen in distilled water (1, 2, 7, 10, 11). Thus the contents of the mitochondria would be released onto the already dried Formvar film and give rise to the pictures obtained.

To test this hypothesis experiments were designed, in order to dehydrate the mitochondria as gently as possible, prior to the drying in high vacuum. Drops of suspensions in distilled water were placed on Formvar films as described above. An initial drying was performed partly at 0° C without special arrangements, and partly with the aid of a current of air dried in a CO_2 alcoholic bath at -80 °C, and then allowed to attain 0° C and finally sucked over the screens in a small glass tube at a slow rate. The shortest drying times amounted to 20 minutes, the longest to 4 hours. All preparations were then evacuated at 0° C in high vacuum, and allowed to attain room temperature *in vacuo*. They were shadow-cast at 30° angle with palladium.

All mitochondria from these preparations presented a rounded form showing only very small membraneous structures at their bases (Fig. 2). No internal structure could be ascertained, nor any finely granular material corresponding to the substance previously observed surrounding the mitochondria. The considerable height of the individual mitochondrion in this type of preparation is indicated by the striking lengths of the shadow, thus giving a three dimensional picture.

Thus, the granular inner body and the finely granulated substance are released from the mitochondria through rupture of the membranes. The above-mentioned substance should be described as a mitochondrial matrix. In distilled water suspensions it must be assumed that the matrix is protected from disintegration by the intact mitochondrial membranes.

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Fig. 1. Mitochondria (mitochondrion?) isolated from 0.88 M sucrose solution, resuspended in distilled water and dried on Formvar film *in vacuo* at 10^{-3} mm Hg. Shadow casting with palladium at an angle of 30°. Original magnification × 4600. *A*—Part of granular inner body. *B*—Mitochondrial membrane. *C*—Matrix substance.

Fig. 2. Same preparation as in Fig. 1 but dried in a current of dried air at 0° C for 60 minutes prior to drying in high vaccum. Note three dimensional, spherical form of mitochondrion. No matrix substance observable. The arrow indicates a small part of the mitochondrial membrane at the base of the mitochondrion.

Experimental Cell Research, **7**, *578–580 (1954)*

MORPHOLOGY OF INTRANUCLEAR INCLUSIONS IN LIVER CELLS INFECTED WITH CONTAGIOUS CANINE HEPATITIS

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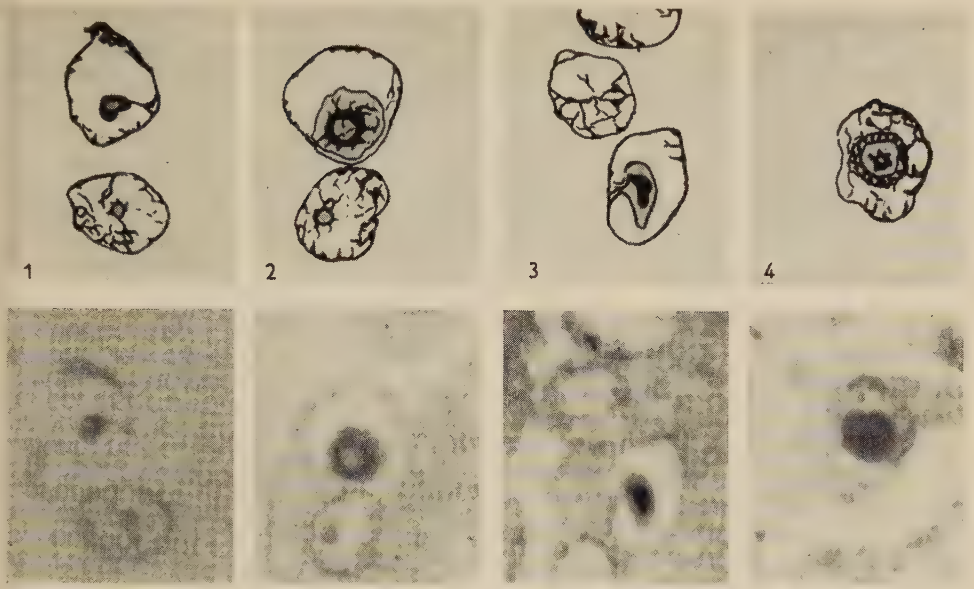
Received July 27, 1954

Caspersson (1) and Hydén (2) have reported that the intranuclear inclusions in skin epithelial cells, infected with *verruca vulgaris*, were formed from desoxyribose nucleotide-containing substances which at first appeared as aggregates around the nucleolus, resembling "the nucleolus-associated chromatin", and gradually coalesced to form large structures. According to their interpretations, the virus acted as a parasite on the nuclear part of the normal protein-forming apparatus of the host cell, in order to fulfill its own needs for reproduction. The mass, thereby produced, was assumed to be a virus mass. Rubarth (3) studied the intranuclear inclusions, characteristic of contagious canine hepatitis (CCH), and viewed the inclusions as manifestation of the localization of the causative virus with little emphasis given to the nucleoli themselves. He noted irregularities in stainability of the inclusions. The hepatitis inclusions gave a positive reaction to thymonucleic acid by Feulgen technique. However, some of them were distinctly basophilic, whilst others more or less acidophilic, indicating that the inclusions contained both baso- and oxychromatin simultaneously, now the one, now the other showing a predominance in different cases. As a rule, the center of the inclusions was more or less markedly basophilic, whereas the periphery had a weaker affinity for stain, regardless of whether it was acidophil or basophil. It appeared that the inclusions were not uniform in structures, but had varied compositions. The present investigations were initiated with the intention of more clearly defining the structures of the CCH inclusions, especially of those at different stages of development, and also of determining the role of the nucleoli in the formation of these inclusions.

From 13 puppies, which died of CCH, small pieces of liver tissue were taken soon after death and fixed in Carnoy solution for tissue sections. The diagnosis, given clinically, was confirmed by post-mortem examinations. The liver cells showed degenerative cytoplasm and swollen nuclei with inclusions, as described by the previous investigator (3). Besides the ordinary tissue preparations for routine examinations, extra-thin sections, about 1 micron in thickness, were prepared from the liver tissue in order to obtain cross-sections of the nuclei and even of the inclusions, so that the internal structures of the inclusions might be observed. The thin sections were stained by the hematoxylin-eosin, Giemsa, and Feulgen methods. The preparations stained with Giemsa were later treated by the Feulgen technique. This permitted a comparative study of the same cells and inclusions stained by these two methods.



Figs. 1–4. Sketches of the liver cells with CCH inclusions with corresponding photomicrographs. Magnification was by Leitz-Wetzlar Periplan 15 × and 2 mm Apo. Oel. Figs. 1 and 2 stained by the Feulgen-light green method and Figs. 3 and 4 by the Giemsa method.

The inclusions, as a rule, were observed around nucleoli. The nucleoli were about normal in size when the inclusion body was small (Fig. 1), markedly enlarged when associated with medium-sized inclusions (Fig. 2), and again small in large inclusions (Fig. 4). The nucleoli were often not visible in particular cross-sections of large inclusions (Fig. 3). Small inclusions consisted of a single layer of basophilic and Feulgen-positive substances (Fig. 1). A similar layer, although much less in quantity, could be observed even in normal liver cells, which picture corresponded to that of nucleolus-associated chromatin (1, 2). The structure of the inclusions became complicated as they grew. Medium-sized inclusions consisted of two distinct layers, surrounding enlarged nucleoli (Fig. 2). The inner layer was of the same characteristics as the layer in small inclusions, but more in quantity. The outer layer was acidophilic, or stained red by the hemotoxylin-eosin method, purplish red by the Giemsa method, and faintly green by the Feulgen-light green method. The basophilic or Feulgen-positive inner layer formed slender processes, branching into the acidophilic outer layer. In large inclusions, the inner layer was comparatively thin, whereas the outer layer became considerably thick. The basophilic and Feulgen-positive branches from the inner layer were densely distributed in the periphery and formed a zone of fine network along the outer boundary of the inclusion (Fig. 4). In other words, large inclusions had three layers around shrunken nucleoli, namely, the basophilic and Feulgen-positive inner layer; predominantly acidophilic intermediate layer; and predominantly basophilic outer layer. Medium-sized inclusions had the inner and intermediate layers around enlarged nucleoli, and small inclusions had only the inner layer. The internal structures were not well demonstrated in thicker liver sections. Large inclusions appeared as basophilic and Feulgen-positive bodies, whereas medium-sized inclusions presented acidophilic bodies with basophilic centers, as noted by Rubarth (3). The nucleus with the inclusion generally was enlarged. The chromatin network was scarce, often leaving wide open areas, especially around the inclusion. Some part of the network were connected with the basophilic and Feulgen-positive branches in the inclusion. In some cases a strand was formed, spun between the inclusion and the marginated chromatin which increased to an extraordinary degree on the nuclear surface (Fig. 1).

On the basis of the above findings, the following interpretations may be given. On responding to CCH infection, nuclei of the liver cells swelled and the basophilic and Feulgen-positive substances around nucleoli increased. The substances gradually accumulated as the nucleoli became larger. The nucleoli reacted to increase their protein-forming activity, as the previous investigators suggested in the case of *verruca* inclusions (1, 2). The substance produced around the nucleoli showed through its staining reactions that it could be composed chiefly of desoxyribose nucleotide. Subsequently, acidophilic substances were produced and formed a layer covering the above formation. The acidophilic layer gradually increased in thickness. A basophilic and Feulgen-positive network increased in the periphery of the acidophilic matrix. By the time the basophilic and Feulgen-positive layer became thinner and the nucleoli became smaller, the protein-forming activity of the nucleoli appeared to decline. Some parts of the basophilic and Feulgen-positive substance could be identical with the virus masses, if the virus consisted chiefly of desoxyribose nucleotide. Some other parts, especially those produced around the nucleoli, could be raw materials to be utilized for virus reproduction.

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Experimental Cell Research, **7**, *581–583 (1954)*

A MORPHOLOGICAL ELEMENT FOUND ON RAT CHROMOSOMES

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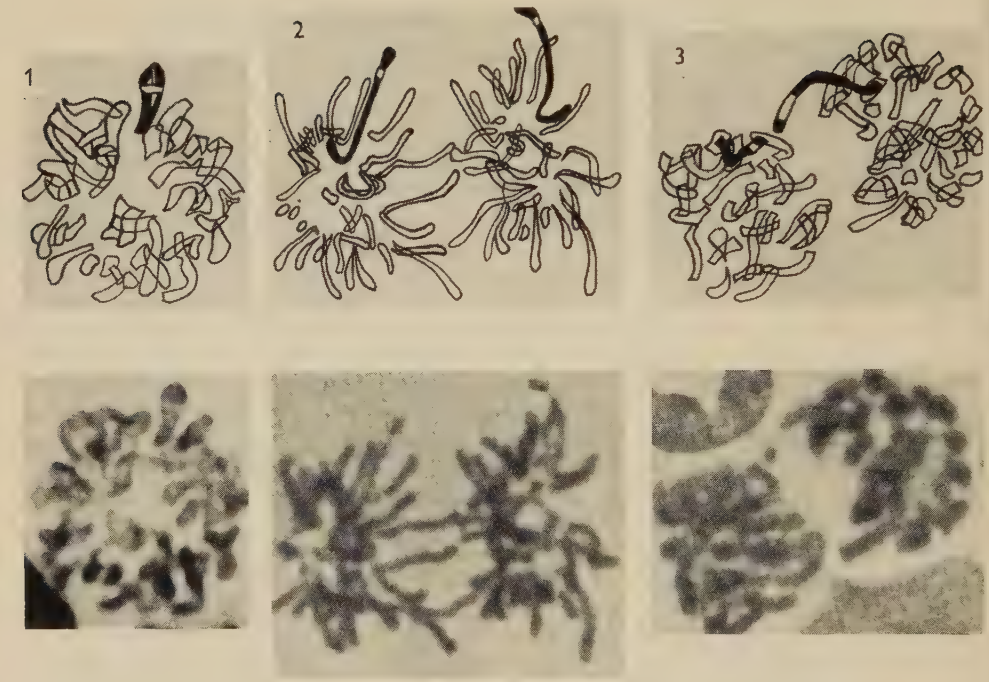
During a recent study of rat myeloid cells in mitosis (3, 4, 5, 6), it was recognized that a portion of a metaphasic chromosome was less azurophilic by the Giemsa method and less deeply stained by the Feulgen technique. Further investigations were carried out by using a new technique which permitted the demonstration of the particular zone more clearly, minimizing altering artefacts which might change the normal forms of the cells, especially of the chromosomes. Such methods as squashing and using a hypotonic solution were avoided.

A piece of a bone marrow, about 2 × 3 × 3 mm, was freshly removed from a normal rat, and rinsed in a few drops of horse serum on an object glass. From the resulting fluid, suspending free cells, very thin film preparations were made, which were quickly dried, fixed first in 1 per cent solution of chromic acid and then in methanol, treated with 1 N solution of HCl for 15 min. at 60° C, immersed in a saturated solution of picric acid for 10 min., and finally stained by Giemsa stain. This technique enabled us to stain only chromosomes and to demonstrate each figure distinctly. The result with Feulgen stain was inferior.

The above-mentioned zone on the metaphasic chromosome was indicated as a chromophobic segment by this method (Fig. 1). The segment was unstained but fairly well defined. Its width was even and equal to the main chromosome body which was interrupted by the segment. The chromophobic segment was present almost constantly in the metaphasic myeloid cells with a near-diploid number of chromosomes, either of the leukoblastic or erythroblastic series. It was usually located at the distal fourth of a large rod-shaped chromosome and was observed in only one member of the pair at any one time. Its incidence and localization were so regular as to suggest that it might be a physiological structure. It is interesting to note that a chromophobic segment of similar characteristics was observed also in Yoshida ascites tumor cells, which are of a reticulum-cell sarcoma cell type.

Chromosomes at several stages of mitosis were similarly investigated. The chromophobic segments were regularly found. At anaphase, the chromophobic segments were observed on two identical chromosomes, large and J-shaped, located symmetrically at each pole (Fig. 2). Each of the chromosomes was undoubtedly derived from a half of the metaphasic chromosome. The chromophobic segment, somewhat slender and longer, was located at the distal fourth on a large chromosome. The kinetochore of the chromosome was at the proximal fourth, as indicated by the bend of the chromosome during its movement toward the pole. At telophase, the chromo-



Figs. 1 3. Sketches of the myelocytes at meta- (1), ana- (2), and telophase (3) with corresponding photomicrographs. Leitz-Wetzlar Periplan 15 × and 2 mm Apo. Oel were used.

somes with the chromophobic segments at similar positions were distributed symmetrically at each pole (Fig. 3). The segment was elongated, probably owing to a greater elasticity of the part, compared with that of the remaining chromosome. The distal end of the chromosome with the chromophobic segment was incorporated during nuclear reconstruction to form daughter nuclei, instead of being cut off and left in the cytoplasm.

Current literature has been explored. A similar chromosomal structure appears never to have been reported in any vertebrate cells.[1] A special zone such as the secondary constriction or SAT zone, however, has been recognized on chromosomes of other kinds of animals and of plants (1, 2, 7). Descriptions of the latter region are similar but differ from the present findings. The chromophobic segment failed to show either a distinctly stained filament or slender trabecule. Interpretations on the chromophobic segment are to be reserved at the present stage of investigation.

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[1] Dr. T. Tanaka, who recently joined our research group, personally informed us of his and Dr. K. Kano's observations which, independently from us, are in progress at Hokkaido University, Japan. They have observed similar unstained zones on chromosomes of male sex-cells of mice, regenerating liver cells of rats, and various ascites tumor cells of rats, which were stained by the Feulgen, aceto-orcein, aceto-dahlia, and aceto-lacmoid methods, combined with a squash technique. The results will be published elsewhere.

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Experimental Cell Research, **7**, *584–588* (*1954*)

CHLOROPLAST STRUCTURE IN GREEN AND YELLOW STRAINS OF *CHLAMYDOMONAS*

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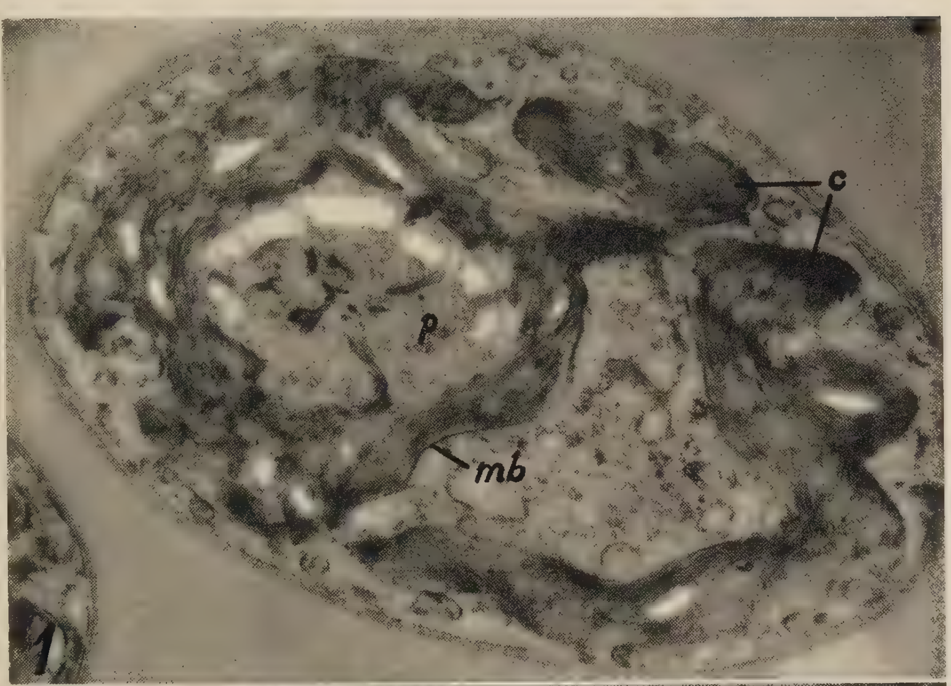
In this note we report preliminary findings made in the course of an electron microscope study of chloroplast structure carried out with normal green strains and pigment-deficient mutants of the unicellular green alga *Chlamydomonas reinhardi*. The presence of a regular, laminated organization in the chloroplast of several light-grown algae has been previously reported (1, 9, 10). It has been further shown by Wolken and Palade that in colorless *Euglena*, grown in the dark, and containing little or no chlorophyll, the large lamellar chloroplasts themselves are absent. When such cells are exposed to light, the chloroplasts reappear.

We have employed a strain of *Chlamydomonas* which forms chlorophyll not only in the light, but also in the dark, where it can utilize acetate as an energy source (6). This normal green strain has been compared with a yellow mutant which differs from it by a unit hereditary factor (5). The yellow strain is green when grown in the light, but yellow when grown in the dark where it, like higher plants, cannot synthesize chlorophyll. The material was fixed in 1 per cent osmium tetroxide buffered at a neutral or alkaline pH with acetate-veronal (3). Best results were obtained between pH 8.0 and 8.5. After embedding in *n*-butyl methacrylate (2), sections of 500 to 300 Å thick were cut (4) and examined without removing the embedding plastic in an RCA electron microscope. Structural differences between the chloroplasts of normal green and of yellow mutant cells, light-grown and dark-grown, will be summarized in this note. The nucleus, mitochondria, elements of the endoplasmic reticulum and other cell features will be described elsewhere.

The normal green strain of *C. reinhardi*, grown in the light, has one large, cup-shaped chloroplast (8) with an undulating surface, a feature which explains the frequent presence, in sections, of several chloroplast fragments. As seen in electron micrographs of which Fig. 1 is an example, the chloroplast is surrounded by a continuous membrane, about 100 Å thick, and contains numerous lamellae similar to those found in other algae and in the grana of higher plants. In addition, the chloroplast membrane encloses the carotenoid-containing eyespot (Fig. 3), the pyrenoid surrounded by plate-shaped, starch-containing bodies (Figs. 1 and 5), and in well-fed organisms, additional starch grains scattered throughout the chloroplast between the lamellae (Fig. 1).

Fig. 1. Light-grown, normal-green *Chlamydomonas reinhardi*. Magnification: 14,600.
Fig. 2. Dark-grown, yellow mutant. Magnification: 40,500.
c, chloroplast; *mb*, chloroplast membrane; *p*, pyrenoid; *s*, starch granules; *e*, eyespot; *li*, lipid inclusion.
Light-grown, normal-green *Chlamydomonas reinhardi*.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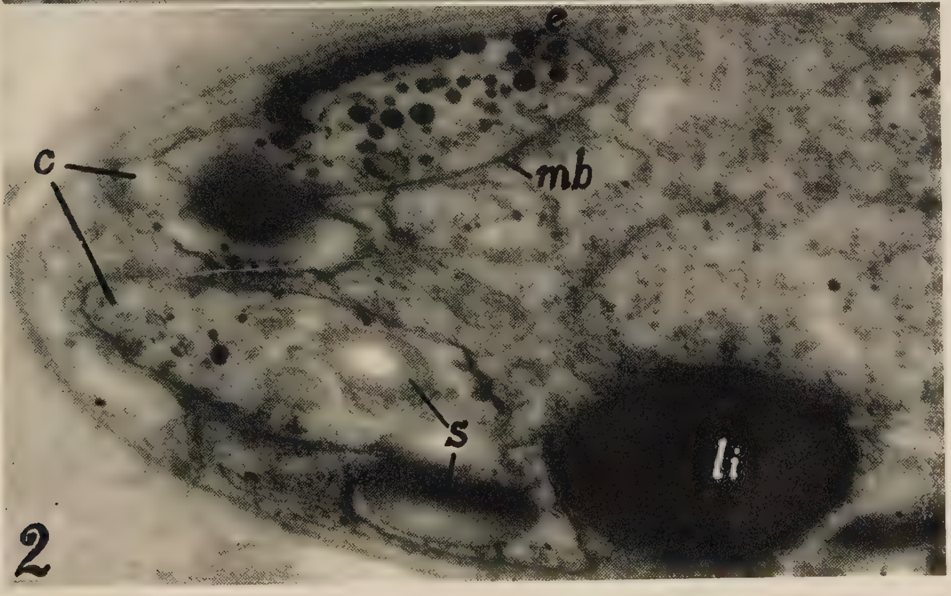
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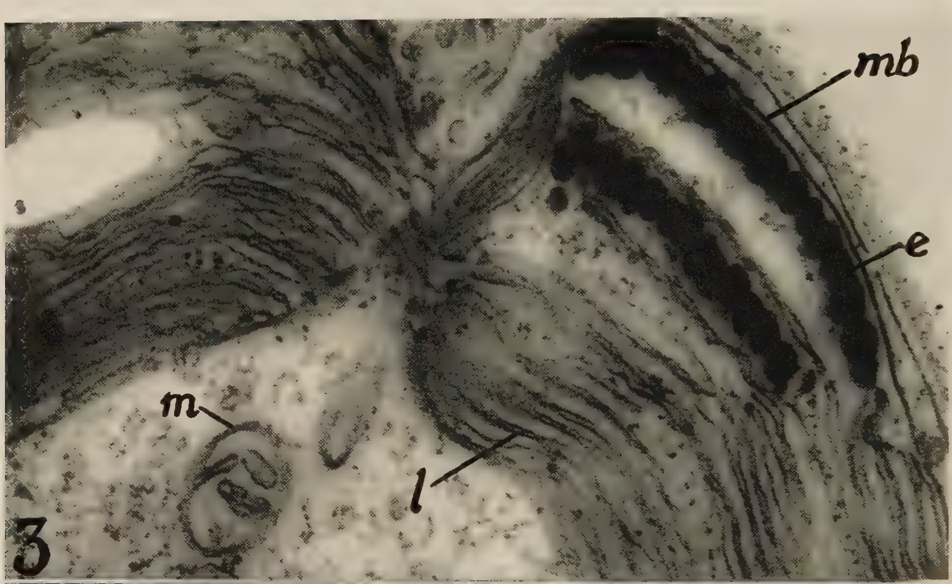

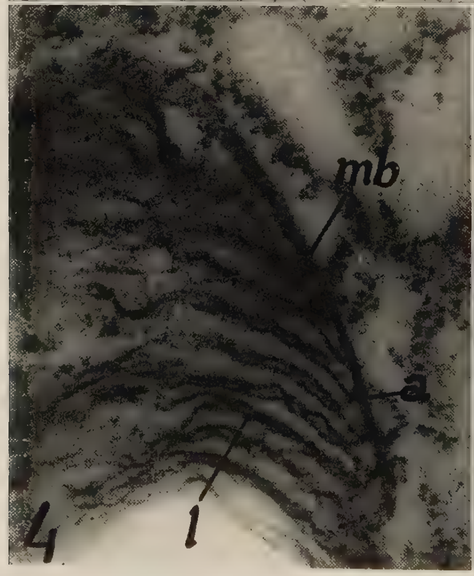

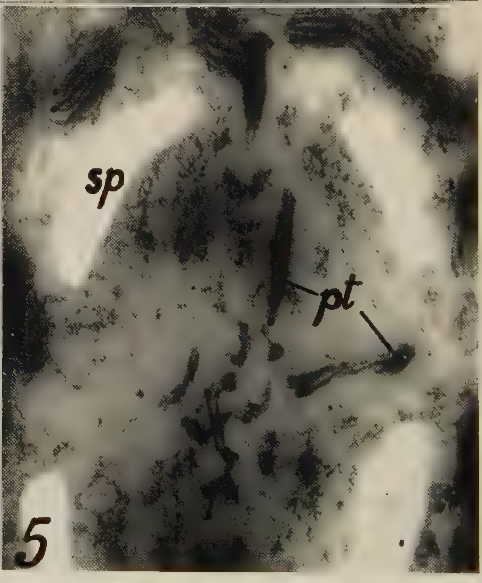

Fig. 3. Detail of the eyespot. Magnification: 54,000.
Fig. 4. Attachment of the chloroplast lamellae. Magnification: 86,000.
Fig. 5. Pyrenoid. Magnification: 29,000.
mb, chloroplast membrane; *l*, lamellae; *a*, point of attachment; *e*, eyespot; *sp*, starch plate; *pt*, pyrenoid tubules; *m*, mitochondrion.

The lamellae occupy most of the chloroplast area and are disposed in tightly packed stacks within which they lie parallel to one another at more or less regular intervals. In contradistinction with the situation found in *Euglena*, the pile of lamellae is not continuous through the entire expanse of the organelle, but is fragmented into small stacks separated by narrow, irregular spaces filled with a fine, granular material comparable with the stroma of higher plant chloroplasts. Though less clearly individualized, the stacks are reminiscent of the grana found in higher plant chloroplasts and in this respect the chloroplast of *Chlamydomonas* may be considered as a transitional form between the agranular and granular types of plastids.

As shown in Fig. 4, the lamellae of the peripheral stacks appear to be anchored to the chloroplast membrane and the form of attachment provides new information concerning the structural unit of the plastid. The lamellae are joined in pairs just proximal to the membrane attachment point; being similarly paired at the other end of the stack, the two joined lamellae with the space in between, form a disc reminiscent of structural units described in retinal rods (7). Each disc measures ~ 340 Å in thickness; ~ 50 Å for each dense lamella and ~ 240 Å for the space separating them. The distance between adjacent discs is ~ 120 Å.

The eyespot, located in an anterior part of the chloroplast just under the membrane, consists of uniform-size, dense spheres hexagonally packed in two parallel layers, separated by two lamellae which at each end of the eyespot are usually continuous with the lamellae of the chloroplast (Fig. 3).

The pyrenoid is a relatively large, non-laminated body about 1.8 μ in diameter, embedded in the posterior part of the chloroplast, partly separated from it by starch plates. Tubular elements, lying between the starch plates, connect the lamellar part of the chloroplast with the pyrenoid (Fig. 1). Within the pyrenoid there exists a network of these tubules, characterized by the presence of irregular thickenings along their walls (Fig. 5).

The morphological features of the chloroplast of normal-green, light-grown cells here summarized are also characteristic of normal green cells grown in the dark, and of the yellow mutant grown in the light. All these cells contain chlorophyll.

However, in the yellow mutant grown in the dark, which contains little or no chlorophyll, profound changes in structure are apparent. The most striking change revealed by electron micrographs, of which Fig. 2 is an example, is the absence of lamellae. In addition, the pyrenoid is not found but the chloroplast membrane is present, and within it are encountered numerous starch grains and the granules of the eyespot less tightly packed than in green cells. Although the lamellae and the pyrenoid are missing, certain structures are present which can be considered as traces of both these elements. In the posterior part of the chloroplast where the pyrenoid of green cells is located, the yellow cells contain bits of those tubular elements with irregular thickenings which we have found to be characteristic of the pyrenoid. The starch plates which by their arrangement delimit the pyrenoid, are absent. Knowing no other features by which to identify the pyrenoid, we cannot say how much of the organelle is present; but only that in the absence of chlorophyll at least certain elements of the pyrenoid are still formed. Between the two layers of eyespot material, instead of the continuous lamellae found in green cells, one finds a row of discrete profiles which represent vesicles or tubules. They are clearly shown in Fig. 2. Similar structures are found in other regions of the chloroplast, especially in proximity to the membrane. These profiles may represent rudiments of the lamellae found in green cells.

Two relationships which emerge from these observations may be noted. First, in both light-grown and dark-grown cells, starch grains are found only within the chloroplast membrane, suggesting that the entire process of starch synthesis from photosynthetically-reduced CO_2 occurs within the chloroplast. Second, it appears that the presence of chlorophyll is necessary for the formation of lamellae. It is tempting to postulate that one of the structural components of the lamellae is represented by chlorophyll itself.

In summary, chlorophyll-containing cells, whether grown in light or dark, contain a complex chloroplast organization consisting of stacks of lamellae, pyrenoid, and eyespot within a limiting membrane. In the absence of chlorophyll, the lamellae are not formed, although the eyespot, starch grains, and certain rudimentary elements possibly related to the lamellae and the pyrenoid are still to be found within the apparently intact chloroplast membrane.

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Experimental Cell Research, 7, 589–591 (1954)

ON THE EFFECT OF ESTRADIOL ON THE DIFFERENTIATION OF THE EGG OF THE SEA URCHIN *PSAMMECHINUS MILIARIS*

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Eggs of *Psammechinus miliaris* were subjected in different stages of development to the action of estradiol (4–8 γ per ml) in sea water. After a treatment for one, two or three hours the eggs were transferred to normal sea water and the development was studied. It turned out very clearly from orienting studies that the eggs have a period of high sensitivity at the beginning of development. In these studies the periods of exposure were usually three hours. The development of eggs with estradiol (4 γ/ml) treated during the period 0–3 hours after fertilization was very pathological, whereas treatment during the periods 3–6 or 6–9 hours after fertilization hardly interfered with the normal development to the pluteus stage. To get more precise information about the period of maximum sensitivity the time of exposure was limited to one or two hours. Table I gives a survey of the results obtained in an experiment of this type. The estradiol concentration was here twice (i.e. 8 γ/ml) that used in the experiments with longer lasting exposures. It follows from Table I that the high sensitivity to estradiol is not yet developed during the first hour of development. The high sensitivity seems to prevail particularly during the 2-8-cell stages. With the formation of the micromeres the period of high sensitivity has passed.

The cleavage and further development was particularly studied in experiments with longer exposures to estradiol of lower concentrations (4 γ/ml). Eggs brought into estradiol immediately after fertilization and left there e.g. for three hours exhibited a rather irregular cleavage with tendency to formation of unequal cells. The hyaline layer often seemed to be damaged. Single cells bulged out from the layer of cleavage cells. After transfer to normal sea water blastulae developed with a somewhat irregular wall. Cells were now seen to leave the epithelial layer and enter the blastocoel. Finally this contained numerous cells or was even completely filled with cells showing signs of degeneration. Nevertheless, the blastulae hatched and exhibited lively swimming movements. Endoderm was rarely formed. When appearing it was rudimentary.

Sometimes 25–40 hours old larvae were found in which no extensive immigration of cells had occurred. The cells present in the blastocoel had a normal appearance. None or at most a rudimentary endoderm developed. Nevertheless, the larvae were bilaterally symmetrical with an oral field surrounded by a ciliary band. No skeleton was present and no arms were formed. Moreover, no stomodeum appeared.

A number of observations indicated that the endoderm material was more or less completely eliminated in these larvae. They resembled in a striking way certain larvae obtained by Hörstadius (2, p. 317). By means of a glass needle he separated the mesomere material + veg_1 from veg_2 + micromeres. Veg_1 corresponds to the

TABLE I

Kristineberg, 6–8 August 1954. Fertilized eggs of *Psammechinus miliaris* were treated for one hour with 8 γ/ml estradiol in sea water. Thereafter they were transferred into normal sea water. Below the development is recorded in per cent of the larvae counted 45 hours after fertilization. The eggs were fertilized on August 6, at 12.30 p.m.[1]

Treatment of the eggs during the following time interval	More than 95 per cent of the eggs were in the following stage when transferred to estradiol	Strongly pathological larvae. Blastocoel mostly filled with pathological cells	Prisms, usually of somewhat irregular form	Plutei	Number counted
1. 12.30–1.30 p.m.	1-cell	27	12.5	60.5	248
2. 1.30–2.30 p.m.	2-cell	94[2]	5.3	1.7	246
3. 2.00–3.00 p.m.	4-cell	100[2]	—	—	300
4. 2.30–3.30 p.m.	8-cell	76	6.0	18	345
5. 3.00–4.00 p.m.	16-cell	14	14	72	256
6. 3.30–4.30 p.m.	32-cell	1	1	98	166

more animal ring of the descendants of the macromeres (2, Fig. 2 f) and belongs to the presumptive ectoderm. The vegetal ring of macromere material, veg_2, represents the presumptive endoderm. The fragment consisting of mesomere material + veg_1 mostly developed into bilateral but purely ectodermal larvae, with an oral field surrounded by a ciliary band. Within this field a stomodeum appeared (2, Fig. 24). Only rarely a small endoderm rudiment appeared. In these cases the cleavage furrow between veg_1 and veg_2 had probably a more vegetal position than usual (2, Fig. 25).

The estradiol treatment applied to the early stages of cleavage thus seems to suppress rather specificly the normal differentiation of the veg_2 and micromere material. The bilateral larvae without endoderm must originate from the more resistant mesomere + veg_1-material.

It is probable that also the ectoderm of the more pathological type with filled blastocoel has developed from the same cleavage cells. If the veg_1-cells were not included into the ectoderm of these defective larvae, an enlargement of the ciliary tuft would have occurred (cf. 2, Figs. 10 and 24). It is known that the ciliary tuft may enlarge even in larvae with strongly pathological vegetal part (6).

There is one important difference between the larvae reared by Hörstadius from mesomere material + veg_1 and the estradiol pretreated larvae with a developed oral field. As already pointed out, no stomodeum was found in these latter. It is well known that vegetal factors are necessary also for the development of the stomodeum (2, 3). Seemingly the treatment with estradiol leaves the inhibition of the ciliary tuft intact, but eliminates nevertheless the influence of the vegetal factors on the stomodeum formation.

Exposure to an appropriate concentration of estradiol in a very early cleavage stage thus exerts a specific action on the endoderm or rather on the endomesoderm.

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[1] In a control in sea water 100 per cent of the eggs developed into plutei.

[2] In *2* and *3* the larvae were of a more pathological type than in *1* and *4–6*.

The elimination of the endoderm is as complete as if the veg$_2$-ring and the micromeres had been separated from the rest of the egg in the 64-cell stage. The chemical elimination occurs in a stage prior to that in which veg$_1$- and veg$_2$-material is separated. Thus a certain localization of the endoderm-forming material seems to prevail already in the 2–8-cell stage. This must not involve that the determination processes with respect to endoderm formation are accomplished in this stage. As known from many experiments it certainly does not involve that the endoderm is definitely segregated, cf. (2, 3). The early localization implies probably that a still reversible determination of the endoderm has been initiated.

The localization of endoderm demonstrated for the early cleavage stages is probably brought about already in the ovary. Retzius (5) has given some figures showing how the surface of attachment of the young oocytes is rather large. The surface of attachment decreases as the oocyte is growing out. There is a possibility that the early attachment delimits a region with certain specific properties which play a role in the vegetal differentiation processes.

If the exposure to estradiol (4 γ/ml) is prolonged, an increasing damage of the endoderm is observed also in stages beyond the "sensitive period". After exposure from 3 to 24 hours to estradiol and transfer to sea water bilateral larvae with none, a reduced or, more rarely, a rather normal endoderm were observed.

Addition of 0.003 M adenosinetriphosphate markedly improved the cleavage in eggs exposed to estradiol. After transfer of the eggs to pure sea water the development was more normal than in eggs which were pretreated with estradiol alone.

Herbst (1) found that in eggs transferred to sulphate-free sea water a pathological development of the endoderm occurs. According to Lindahl's (4) ingenious explanation a poisonous substance is formed in the vegetal part of the sea urchin embryo, but this substance is detoxicated by its esterification with sulphate.

In varied experiments it was shown that the exposure to estradiol in sulphate-free sea water is much more harmful to the eggs than the exposure to this sustance dissolved in sea water containing sulphate. Eggs exposed to estradiol (4 γ/ml) in sulphate-free sea water 3–6 hours after fertilization are considerably injured. As mentioned above an exposure during this period to estradiol in normal sea water has none or a slight effect. The presence of sulphate in the sea water thus considerably detoxicates the poisonous effect of estradiol.

The pathological changes of the endoderm caused by sulphate-free sea water alone are only observed in the gastrula stage (1, 4). Nevertheless the question arises whether the effect of sulphate-free sea water may be due to the release of substances related to estradiol. It is not excluded that such substances play a role in the vegetal determination processes and in gastrulation.

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Experimental Cell Research, 7, 592–594 (1954)

HISTOCHEMICAL ALTERATIONS IN SUCCINIC DEHYDROGENASE ACTIVITY OF GUINEA PIG TISSUES FOLLOWING ADMINISTRATION OF DIPHTHERIA TOXIN

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Pappenheimer and Hendee (1) have presented evidence that diphtheria toxin is closely related to the protein moiety of diphtherial cytochrome *b*. As a tentative hypothesis they proposed that diphtheria toxin may act by interfering with the normal function of cytochrome *b* or some intimately associated enzyme in the tissues of the susceptible host. Later (2), these investigators concluded that succinic dehydrogenase and cytochrome *b* may well be identical both in diphtherial extracts and in ox-heart preparations. Although this identity may be questioned, these successive components of succinoxidase system are firmly attached to the framework of mitochondria. Since the form and distribution of mitochondria is altered by diphtheria toxin (3) it was found advisable to approach the question by employing a histochemical method for succinic dehydrogenase (4). In this method a tetrazolium salt acts as hydrogen acceptor for succinic dehydrogenase. When reduced it becomes deposited as colored granules of formazan at the sites of activity.

Twelve guinea pigs were injected subcutaneously with 10 M.L.D. of a stable diphtheria toxin (M.L.D. 0.012 ml, Lf 25).[1] Another twelve animals of the same sex and weight served as untreated controls. All the animals were fasted during the experiment. After six hours, four of the intoxicated animals and their respective controls were killed by a blow on their head and bled out. Another four were sacrificed twelve hours after the injection, and the remaining four were killed in a moribund state after 20 to 28 hours. Immediately after decapitation pieces from the liver, kidney, femoral and cardiac muscle were frozen and sectioned simultaneously with a corresponding control specimen at 30 μ. The frozen sections were dipped directly from the cooled knife into incubation vials kept at 37° C. The incubation medium was prepared according to Seligman and Rutenburg (4), except that neotetrazolium was used instead of blue tetrazolium. The incubation time for liver sections was 30 minutes and that for sections from kidney, skeletal muscle and heart 15 minutes.

In liver of the control animals the purple formazan was deposited as small needle-like or spheroidal granules in the periportal cells of the liver lobules (Fig. 1 a). Six hours of diphtheric intoxication was not followed by any appreciable change in the formazan pattern, whereas after twelve hours the formazan deposition becomes more scarce and the granules tended to fuse in conglomerates (Fig. 1 b). This agrees well with the behavior of liver mitochondria in diphtheric intoxication (3).

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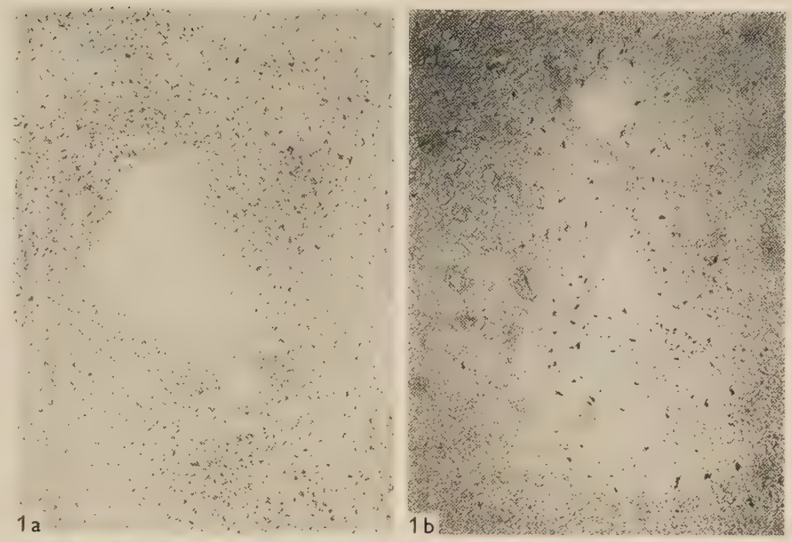
[1] Kindly supplied by Lääketehdas Orion Oy., Pharmaceutical Manufacturers.



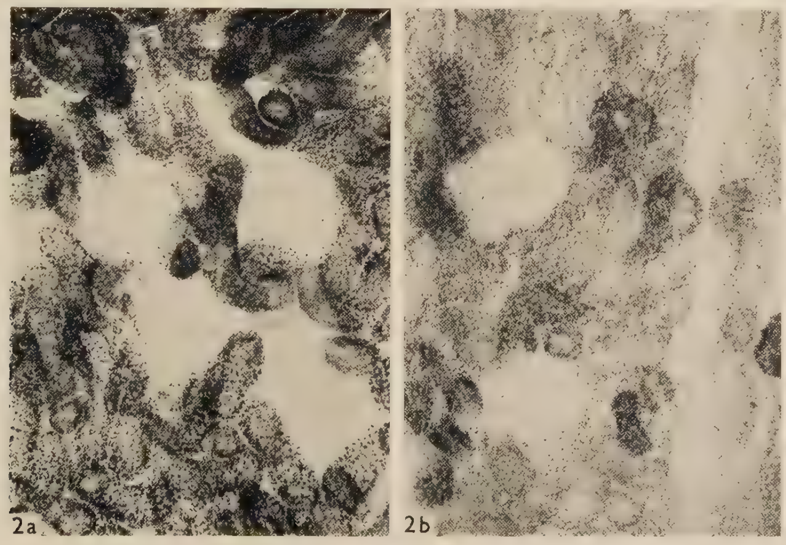


Fig. 1 A, Formazan granules in normal guinea pig liver indicating distribution of succinic dehydrogenase activity. — B, 20 hours after administration of 10 M.L.D. diphtheria toxin. Note the sparse deposition of formazan granules and their fusion to clumps. Lipid droplets pink of formazan are also seen. Magnification × 300, reduction $^1/_2$.

Fig. 2 A, Normal activity of succinic dehydrogenase in kidney cortex of guinea pig. — B, 12 hours after 10 M.L.D. of diphtheria toxin. Magnification × 300, reduced $^1/_2$.

In the cells of kidney tubules the number and size of formazan granules progressively decreased in the course of diphtheric intoxication (Figs. 2 a and b). This alteration was noticeable already six hours after the injection of the toxin.

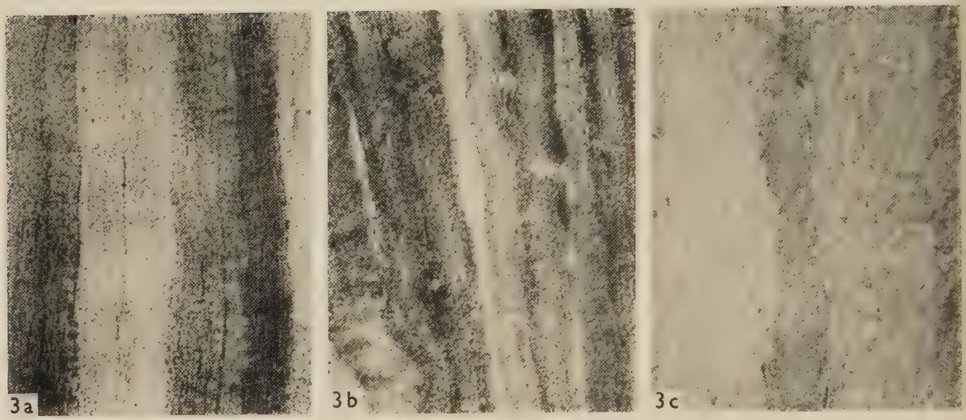


Fig. 3 A, Succinic dehydrogenase activity in a femoral muscle of a normal guinea pig. Note the variability of staining of the fibers. — B, 12 hours after 10 M.L.D. of diphtheria toxin. Note the somewhat increased granulation and the more uniform staining. — C, 24 hours after 10 M.L.D. of diphtheria toxin. The animal was paralyzed. Only one fiber in the picture exhibits a sparse granulation. Magnification × 300, reduced $^1/_3$.

In skeletal muscle the administration of diphtheria toxin was followed within twelve hours by a transient increase in the formazan granulation resulting in a more monotonous staining picture (Figs. 3 a and b). When the animals became paralyzed there was a marked reduction in the deposition of formazan (Fig. 3 c).

The cardiac muscle displayed the highest succinic dehydrogenase activity as estimated from the most dense and rapid appearance of formazan granules. In the control animals the resulting coloration was brownish purple, whereas in all stages of diphtheric intoxication the staining effect was accentuated by a bluish tone. Whether the bluish purple color actually indicates increased activity of succinic dehydrogenase will be checked with quantitative methods. It is interesting that the activity of heart did not seem to be reduced in the moribund animals. Further studies are now in progress.

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Experimental Cell Research, 7, 595–596 (1954)

INTRACELLULAR DISTRIBUTION OF DEHYDROGENASE ACTIVITY IN *C. DIPHTHERIAE* AND IN *C. PSEUDODIPHTHERITICUM*

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When studying the occurrence of Krebs' citric acid cycle in *Corynebacterium diphtheriae*, one of us (L. J.) found that malonate had no effect on the dehydrogenation of acetate by fresh cells of the diphtheria bacterium, whereas in lyophilized cells and in cells disrupted by sonic treatment this reaction appeared to be malonate-sensitive. The dehydrogenation of succinate was inhibited by malonate regardless of the state of the cells (4). This could be explained if the respective enzyme systems were localized in the bacterial cell and if acetate and, on the other hand, succinate and malonate had different possibilities to reach these sites of enzymic activity.

This assumed localization of dehydrogenase activity in some *Corynebacteria* is now substantiated by experiments in which neotetrazolium chloride (NT) was used as hydrogen acceptor. NT, when reduced by living cells, is deposited at the sites of dehydrogenase activity as insoluble, purple formazan, thus making these sites microscopically discernible (2). This quality of tetrazolium salts has been widely used in the study of the localization of dehydrogenative activity in various cells, the bacteriological applications hitherto published having recently been reviewed by Mudd (5).

The *Corynebacteria* studied were *C. diphtheriae* (the CN 2000 variant of Park-Williams 8 strain, and local strains of the *gravis, mitis* and *intermedius* types) and *C. pseudodiphtheriticum*. The bacteria, except the *intermedius* type, were cultivated on a modified dilute caseinhydrolysate medium (3) containing 2 per cent agar. Instead of maltose, 1 per cent (w/v) sodium acetate trihydrate was used as the carbon source. The *intermedius* type was cultivated on Loeffler slants. Twenty-four hour cultures of each strain (incubated at 34° C) were harvested and suspended in 0.2 M phosphate buffer of pH 7.2. The suspensions were diluted to the same opacity (tube No. 8 in the Wellcome series of opacity tubes), and then used in the experiment.

The experiments were carried out in test tubes at room temperature: 2 ml of the bacterial suspension, 1 ml of 0.1 M substrate solution or—in the test for endogenous dehydrogenation—1 ml of 0.1 M phosphate buffer were mixed, and then 1 ml of 0.05 per cent (w/v) solution of NT in water was added. The substrates (acetate, α-ketoglutarate, and succinate) were used as neutralized solutions of the free acids. The final concentrations were thus: substrate 0.025 M, buffer 0.1 M, and NT 0.0125 per cent; final pH 7.2, final volume 4 ml.

Five identical tubes were used for each test; the reaction was stopped by adding 0.5 ml of neutral 38 per cent formalin into the tubes after 5, 10, 15, 30 and 60 minutes, and the macroscopical and microscopical staining reactions were compared.

A strong dehydrogenation of endogenous substrates was apparent in the *gravis* and CN 2000 strains, as displayed by the intensive and rapid staining of the cells in tests without added substrate. The staining of *C. pseudodiphtheriticum* was less intense and that of the *mitis* type clearly weaker. The reduction of NT by the *intermedius* type was hardly discernible. This divergent behaviour of the *intermedius* type may, at least partly, be derived from the fact that, being unable to grow on the casein-hydrolysate acetate agar, it was cultivated on a different medium than the other strains.

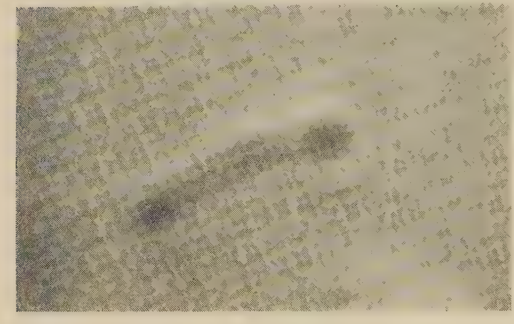

Fig. 1. *C. diphtheriae*, Park & Williams 8 strain, CN 2000 variant. Bipolar deposition of formazan indicating the sites of dehydrogenase activity. Final magnification × 7500.

Microscopical examination revealed, in all the strains studied, that the purple formazan was distributed intracellularly as clearcut polar and/or central granules, with the other parts of the cell remaining unstained (Fig. 1). This staining pattern with NT was in good agreement with the darker-stained areas and the metachromatic granules observed in the bacteria of respective samples after treatment with methylene blue and with Neisser's differential stain. Furthermore, a similar distribution of staining was observed when Janus Green B (0.01 per cent (w/v) in water) and Bennett's reagent (1) for sulfhydryl groups (sat. sol. in abs. ethanol) were used. The granules stained blue green and red, respectively, whereas the other parts of the cell were lighter-coloured.

The effects of added substrates (acetate, α-ketoglutarate, and succinate) were then studied. Acetate accelerated most markedly the rate of staining. This could be observed in all *Corynebacteria* tested, but it was most pronounced in the *mitis*, *gravis*, and CN 2000 strains of *C. diphtheriae*. It is interesting that in fresh cells of the CN 2000, succinate only slightly accelerated the reduction of NT, less than α-ketoglutarate, whereas in lyophilized cells the effects were reversed. The addition of the substrates caused no discernible change in the distribution and number of the formazan-stained granules in single cells. The size of the granules, however, seemed to be somewhat increased and their colour intensified. Likewise the addition of substrates seemed to increase the number of stained bacteria in each sample.

The results suggest that in the *Corynebacteria* studied the enzyme systems concerned in the dehydrogenation of acetate, α-ketoglutarate, and succinate seem to be localized or concentrated in definite cellular inclusions. There apparently exist differences in the accession of the tested substrates to these structures which are evidently identical with the areas made discernible by methylene blue, Neisser's stain, Janus Green B, and the sulfhydryl reagent. According to Mudd (5), these structures may be regarded as equivalents of mitochondria in higher organisms.

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A SET OF INSTRUMENTATION FOR GENERAL QUANTITATIVE CYTOCHEMICAL WORK BY HIGH RESOLUTION MICROSPECTROGRAPHY

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So many different groups of chemical substances are theoretically and practically accessible for "direct" microspectrography (work in the unbroken cell structure) that the method might be the basis for a fairly general quantitative cytochemical approach, see Fig. 1. By combination with isolation procedures ("indirect" microspectrography, ultramicrocolorimetry), other substances unsuitable for "direct" microspectrography can also be examined. The most promising field of application in general seems to be the protein metabolic processes concerned in cell growth.

The properties of the average biological material make the following two conditions assume primary importance for the construction of any type of microspectrographic instrumentation. Without the fulfilment of these, no work of quantitative significance can be carried out.

1. All work must be done with high resolution because of the inhomogeneities in the cellular structure. The resolution required varies with different objects, but 1 μ can be mentioned as the largest size of the individual measuring spot.

2. Especially in the ultraviolet region, the irradiation of the object must be minimized in order not to injure the object by irradiation. With the various photelectric cells available at present this means that one must work near the theoretical limit of the measurement.

In the optical spectral range, methods fulfilling these conditions have been developed long ago for the measurement of individual spots in the preparation. In the X-ray region the first critical condition, 1, was recently fulfilled by the apparatus developed by Lindström (1).

The factors which have until recently seriously limited the possibility of applying these methods to general cell metabolic problems on large scale are the exceedingly time-consuming character of the most important phase of the work, i.e. the determination of the total contents of a certain substance in a defined part of a cell or a tissue, and also the relative difficulty in doing high resolution work with the equipment earlier available. It could be estimated that a 100-fold increase in the speed of work would make it possible to make enough observations on inhomogeneous populations to allow statistical determination. Therefore we have developed a microspectrograph (2, 3, 4) which, with the aid of an automatic scanning device and an automatic computer, gives the desired increase in speed of measurement. This

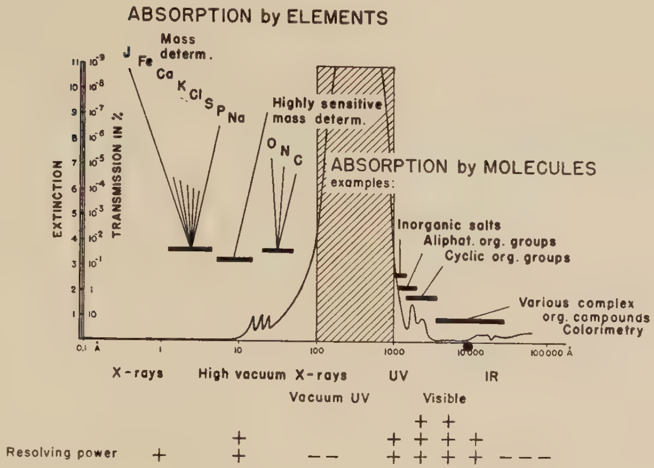
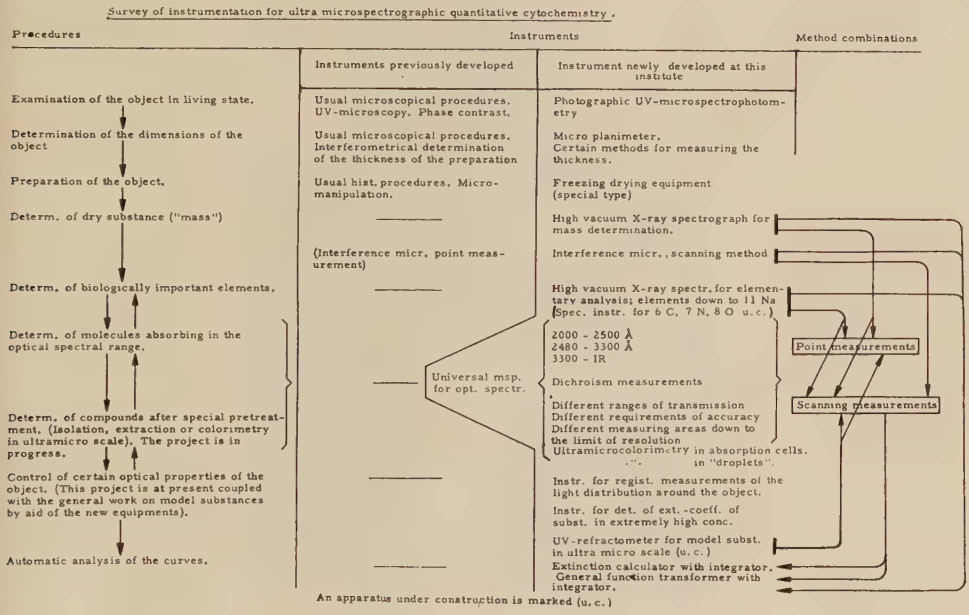


Fig. 1.


Survey of instrumentation for ultra microspectrographic quantitative cytochemistry.

Procedures	Instruments previously developed	Instrument newly developed at this institute	Method combinations
Examination of the object in living state.	Usual microscopical procedures. UV-microscopy. Phase contrast.	Photographic UV-microspectrophotometry	
Determination of the dimensions of the object	Usual microscopical procedures. Interferometrical determination of the thickness of the preparation	Micro planimeter. Certain methods for measuring the thickness.	
Preparation of the object.	Usual hist. procedures. Micromanipulation.	Freezing drying equipment (special type)	
Determ. of dry substance ("mass")	—	High vacuum X-ray spectrograph for mass determination.	
	(Interference micr. point measurement)	Interference micr., scanning method	
Determ. of biologically important elements.	—	High vacuum X-ray spectr. for elementary analysis; elements down to 11 Na (Spec. instr. for 6 C, 7 N, 8 O u.c.)	
Determ. of molecules absorbing in the optical spectral range.	Universal msp. for opt. spectr.	2000 - 2500 Å 2480 - 3300 Å 3300 - IR Dichroism measurements	Point measurements
Determ. of compounds after special pretreatment. (Isolation, extraction or colorimetry in ultramicro scale). The project is in progress.		Different ranges of transmission Different requirements of accuracy Different measuring areas down to the limit of resolution Ultramicrocolorimetry in absorption cells. " in "droplets".	Scanning measurements
Control of certain optical properties of the object. (This project is at present coupled with the general work on model substances by aid of the new equipments).	—	Instr. for regist. measurements of the light distribution around the object. Instr. for det. of ext.-coeff. of subst. in extremely high conc. UV-refractometer for model subst. in ultra micro scale (u.c.)	
Automatic analysis of the curves.	—	Extinction calculator with integrator. General function transformer with integrator.	

An apparatus under construction is marked (u.c.)

Fig. 2.

instrument has proved very suitable for routine work on a great variety of different biological materials during the last three years. Therefore, we have tried to develop a complete set of instruments which will be of general use for quantitative cytochemical work (Fig. 1). A series of instruments listed in the right column of Fig. 2 have been constructed. In order to save instrumentation and working time, many parts of the different instruments are interchangeable. The most expensive and technically complex parts of the equipment are assembled in a "central instrument" (universal microspectrograph, Fig. 2). This instrument is modified for different special tasks by additional equipment. By these means, all scanning measurements for optical as well as X-ray microspectrography and also scanning interference microscopy can be carried out in the universal microspectrograph. In addition, the "function transformer" is nowadays designed so as to be directly attached to the universal microspectrograph and automatically recalculates the measured values into functions which are proportional to the contents of the substance being studied. It also registers the running integral of the latter function. A detailed description of the complete set of instruments will be published later, and the central instrument—the universal spectrograph—will be commercially produced.

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Experimental Cell Research, **7**, *601–602 (1954)*

A SCANNING INTERFERENCE MICROSCOPE ARRANGEMENT

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The excellent possibilities for the application of interference microscopy for the determination of the dry weight of individual cells, or parts of cells, have been clearly demonstrated by Davies and collaborators (5, 6). However, it is necessary to eliminate or compensate for the non-homogeneous nature of most cells. This problem is exactly the same as that which led to the development of automatic procedures for microspectrophotometry.

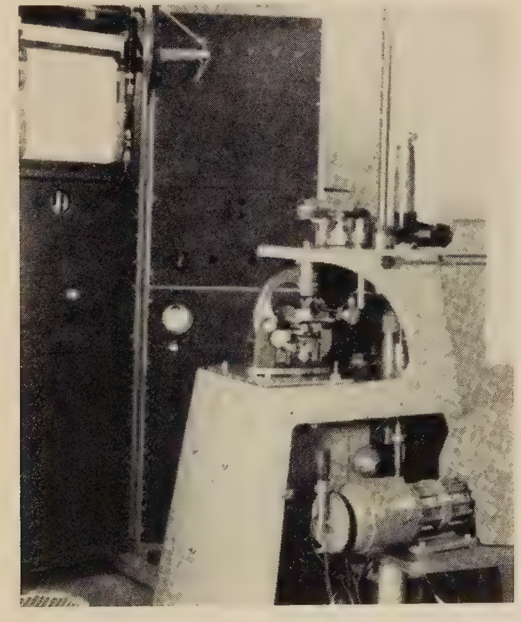

Fig. 1.

Thus, the scanning optical "universal microspectrograph" described earlier (1, 2, 3, 4), when integrated with a suitable interference microscope, can also be applied to scanning interference measurements. Fig. 1 illustrates a Baker interference microscope adapted to the microspectrograph described in this journal ((4) Fig. 1).

Using the instruments described, Fig. 2 shows the course of a registered curve through a microscopic glass sphere imbedded in a medium having a slightly different refractive index than the glass sphere. The curve represents the central part of the sphere where the optical path differences are about 1 λ, which is the highest value to be expected in an average biological preparation. The curve is directly copied from the registration paper and some theoretically calculated values are pointed out. The agreement between the theoretical and measured curve is satisfactory for biological work: 2 per cent error. This error may be due partly to the irregular shape of the test object.

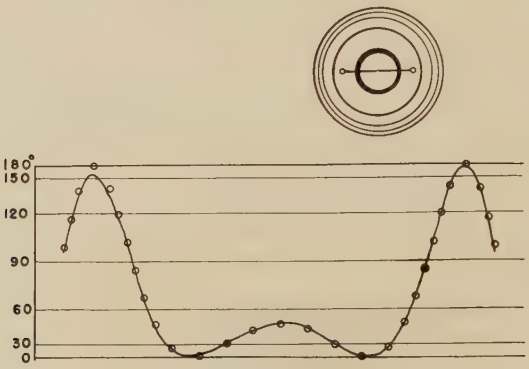

Fig. 2.

From the results so far obtained, scanning interference microscopy, combined with the electronic computer evaluation of the measured curve (7), has proved to be a practical and important addition to the general arsenal of methods for quantitative cytochemical work.

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A GENERAL FUNCTION-TRANSFORMER FOR MICROSPECTROGRAPHIC USE

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In ultramicrospectrography, with quantitative cytochemical aims, the time gained by automatic procedures is of decisive importance for the practical application of these methods to average cell metabolic problems.

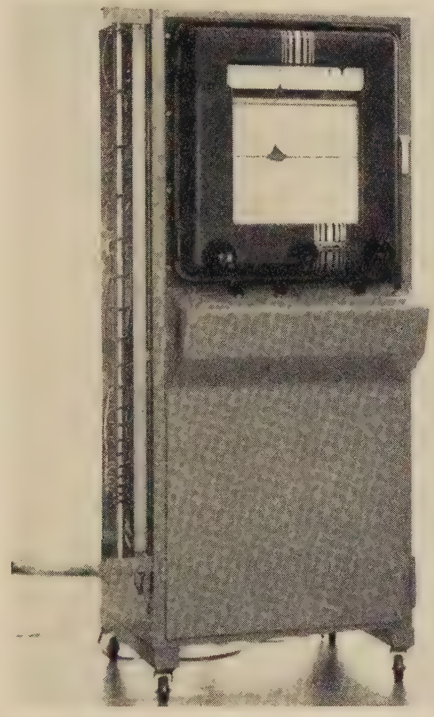
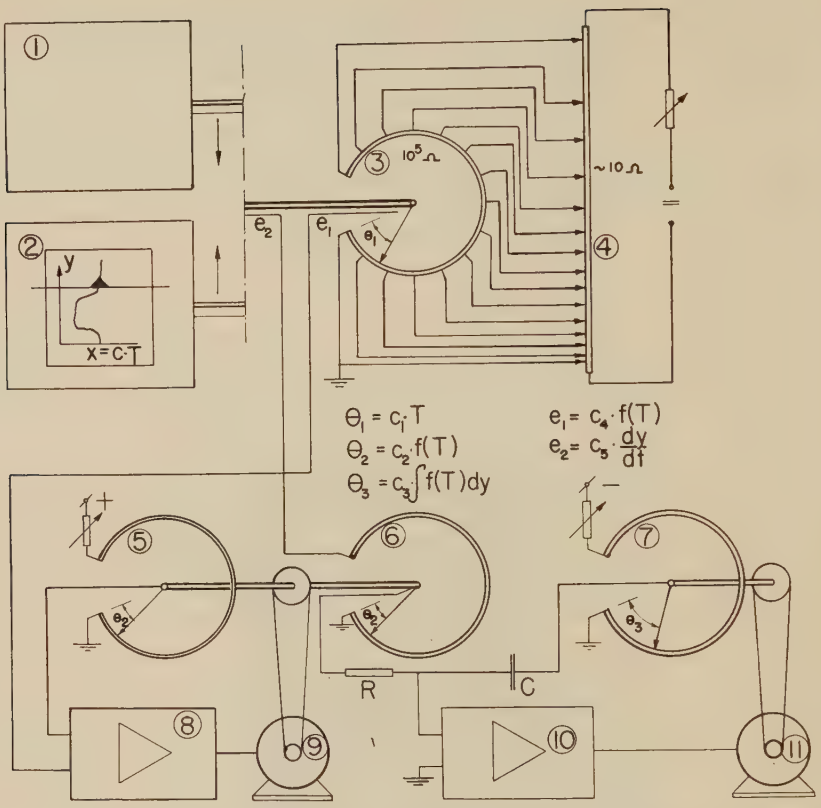


Fig. 1.

A computer has earlier been described for the evaluation of transmission data from the scanning ultramicrospectrograph (1, 2, 3).

The most recent model of the universal microspectrograph (4) is constructed to do scanning measurements on photographic plates from X-ray microspectrographs,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y
X=C·T
③
$10^5 \Omega$
$\sim 10 \Omega$
④
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e_1
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$\theta_1 = c_1 \cdot T$
$\theta_2 = c_2 \cdot f(T)$
$\theta_3 = c_3 \int f(T)dy$
$e_1 = c_4 \cdot f(T)$
$e_2 = c_5 \cdot \frac{dy}{dt}$
+
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θ_2
θ_2
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R
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⑧
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⑩
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Fig. 2. — 1. Microspectrograph. 2. Hand-operated indicator. 3, 4. Function transforming unit. 5, 7; 8, 10; 9, 11. Speedomax X_1-X_2-recorder. 6. Multiplying potentiometer. Transformed function registered by pen governed by 5; integral of transformed function by 7.

or optical spectrographs, and to be used with the scanning interference microscope arrangement. Therefore there was a need for a computer in which the measured data could be recalculated after an arbitrary function.

The instrument described below was constructed. The measurements data may be fed into the instrument in either one of two ways: (i) the data are fed in during the actual run by connecting the instrument directly to the microspectrograph, or (ii) the recorder paper of a measured curve may be placed in the transformer and the curve may be followed by a hand-operated indicator which in turn drives a potentiometer. The instrument records the desired transformed function and the running integral of this function on the same paper. When calculating extinction coefficients in optical spectrography the transformation function is a simple logarithmical one. On photographic material it is an empirically found function and in scanning interference microscopy it is a trigonometrical function. Fig. 1 shows the general appearance of the instrument and Fig. 2 gives the main details of its construction.

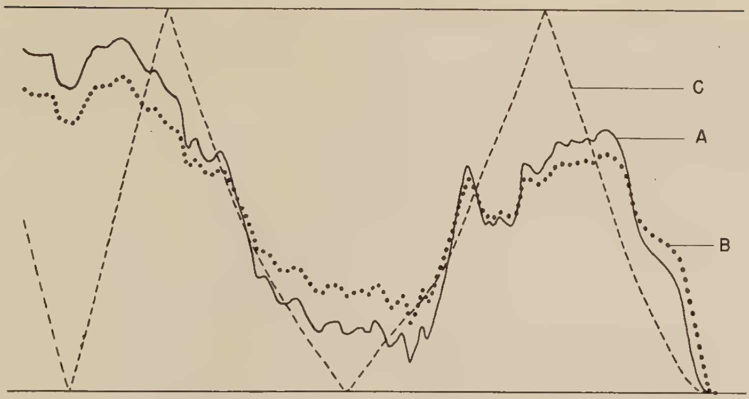

Fig. 3. *A*. The original curve, *B*. The transformed curve, *C*. The integral of *B*.

The speed of the computer is so great that within 2 per cent accuracy it is capable of following even the fastest run possible in the spectrographs. The limiting factor is the time constant of the pen writing the transformed function. In order to permit slow runs of extremely complicated inhomogeneous materials for which very long running time in the spectrograph is necessary, the electrical integrator is constructed in such a way that the integration is independent of time.

Fig. 3 shows a curve transformed according to the trigonometrical function used in scanning interference microscopy.

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Experimental Cell Research, 7, 606–608 (1954)

KRISTALLGITTERSTRUKTUR DES GRANUM JUNGER CHLOROPLASTEN VON *CHLOROPHYTUM*

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Eingegangen am 4. Oktober, 1954

Durch elektronenmikroskopische Untersuchungen erwachsener Chloroplasten der Phanerogamen ist die lamellare Struktur der Grana (1, 3, 5, 6) wie des Stroma (1, 5, 7) endgültig gesichert. Entsprechende Untersuchungen an jüngsten und heranwachsenden Chloroplasten führte bis jetzt nur Leyon durch (4, 5). Er kommt zu dem Ergebnis, dass die Stromalamellen im jungen Chloroplasten, zuerst in geringer Zahl, auftreten. Über die Struktur der Grana konnte kein klares Ergebnis erhalten werden.

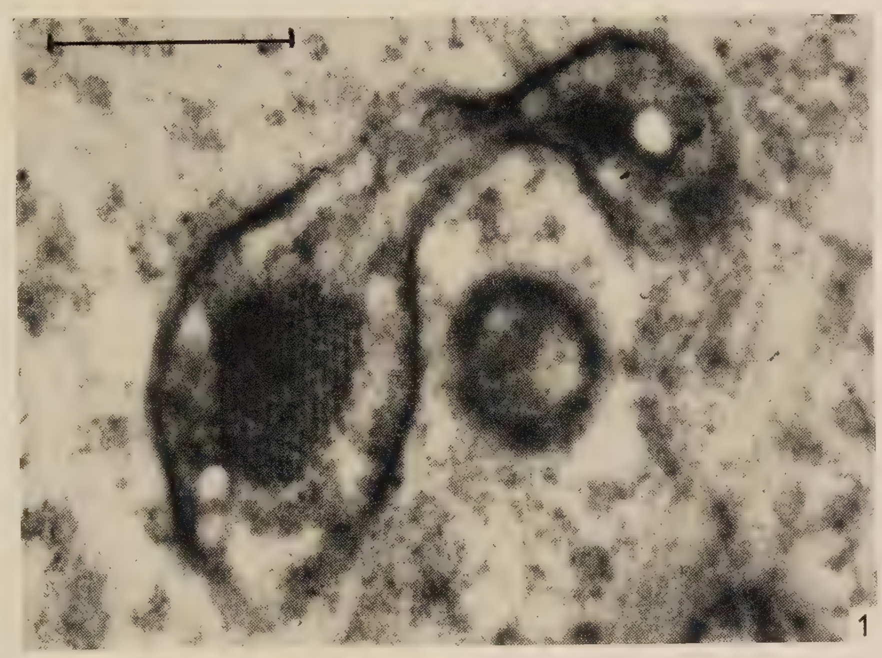
Zur Aufklärung der Struktur junger Chloroplasten fixierte ich möglichst junge, 1–3 cm lange, noch kaum bleich-gelblich grüne Blätter einer 2½ Tage vorher verdunkelten *Chlorophytum*-Art. Die ausgewachsenen Blätter werden 24 cm lang und sind über die ganze Blattfläche gleichmässig dunkelgrün gefärbt. (Fixierung bei Zimmertemperatur mit 1%iger OsO_4 in Veronalpuffer, pH 7, 2. Nach gründlichem Auswaschen und anschliessender Entwässerung Einbetten in Metacrylatgemisch. Schneiden mit dem Ultramikrotom nach Sjöstrand mit nach den Angaben des Autors geschärften Rasierklingen. RCA EMU 2c Elektronenmikroskop.)

An Schnitten von genügender Dünne lässt sich in den Grana eine klare und sehr regelmässige Folge parallel geordneter Reihen erkennen (Abb. 1 und 2). Zunächst war die Deutung derselben als senkrecht durchschnittene Lamellenstruktur, wie sie von den Grana ausgewachsener Chloroplasten bekannt ist, am nächstliegenden. In zweifacher Hinsicht ist jedoch das Muster von dem in den Grana erwachsener Chloroplasten verschieden: Jede Reihe zeigt eine Differenzierung in dunklere, kreisrunde „Flächen“, die in sehr regelmässigen Abständen mit etwas helleren, durch parallele Linien begrenzte Flächen miteinander wechseln. Da die Reihen nicht nur parallel zueinander sondern auch in Bezug auf die geschilderte Differenzierung zueinander geordnet liegen, ergeben sich Querreihen, so dass eine deutliche Gitterstruktur zustande kommt. Etwas analoges ist von der Reihenstruktur, die in den Grana ausgewachsener Chloroplasten gefunden wurde, nicht bekannt. Man vergleiche die sehr klaren Bilder, wie sie Leyon (5), sowie Steinmann und Sjöstrand (7) erhalten haben. Völlig abweichend von dem Muster erwachsener Grana ist der weitere Befund: An ein solches Gitter kann sich seitlich ein zweites ansetzen, in welchem die — unter sich parallelen Reihen — in einem Winkel zu dem ersten verlaufen (Abb. 2). Es konnten bis zu 5 in dieser Weise verschieden zueinander liegende Gitter festgestellt werden.

---

[1] Permanente Adresse: Neubadstrasse 133, Basel, Schweiz.

Junge Chloroplasten von *Chlorophytum*. Elektronenmikroskopische Vergr. 4050 ×. Die 2 Kopien verschieden stark vergrössert. Die Striche geben die Länge von 1 μ an.



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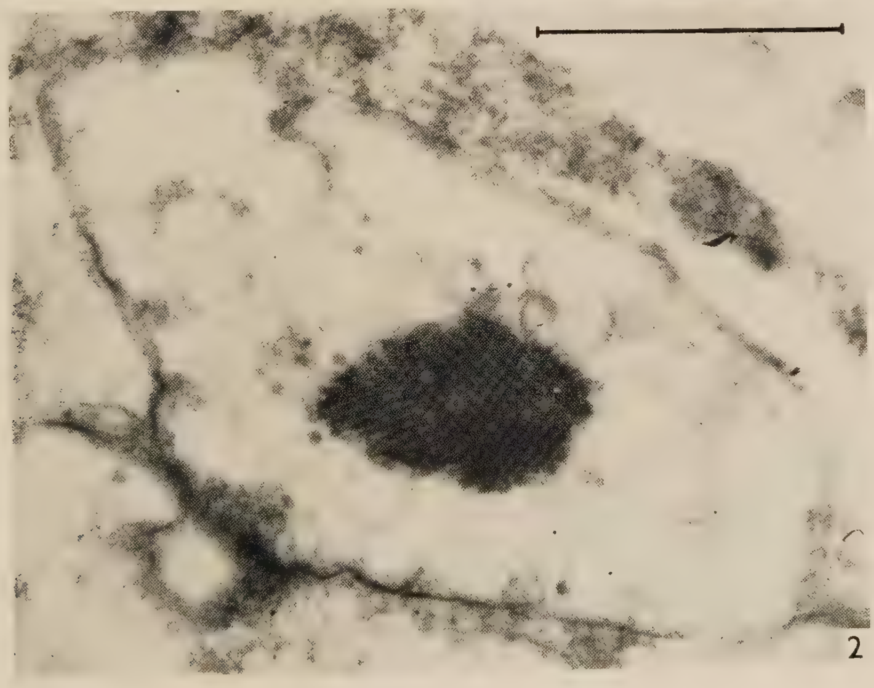

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Das Granum der jungen Chloroplasten besteht demnach nicht aus parallel liegenden Lamellen, sondern aus einem oder mehreren aneinander sitzenden Kristallen, deren Kristallgitter — je nach der Lage der Kristallachsen eventuell in verschiedenen Richtungen getroffen — elektronenmikroskopisch an Dünnschnitten nachweisbar sind. Es wird nunmehr klar, wie in jungen, zunächst homogen rot fluorescierenden Chloroplasten intensiv rot fluorescierende Grana auftreten (2): Bei Erreichung einer bestimmten Concentration des im Stroma des Chloroplasten gebildeten Chlorophylls kommt es infolge von Kristallisation zur Bildung der Grana, der Bildung von Stärke aus Zucker vergleichbar.

Auf die zahlreichen und sehr verschiedenartigen Fragen, die sich aus dem Nachweis der Kristallnatur des jungen Granum im Chlorophytumblatt ergeben, soll erst in der ausführlichen Arbeit eingegangen werden. In dieser findet man auch sämtliche photographischen Belege.

Herrn Dr. Sjöstrand danke ich für die grosse Gastfreundschaft in seiner Abteilung des Anatomischen Instituts, sowie allen anderen, die mich durch Rat und Tat unterstützten, herzlich. Dem Präsidium des Schweizerischen Nationalfonds, welches mir den Aufenthalt in Stockholm ermöglichte, bin ich ebenfalls zu Dank verpflichtet.

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Experimental Cell Research, 7, 609–611 (1954)

THE STRUCTURE OF CHLOROPLASTS

VI. THE ORIGIN OF THE CHLOROPLAST LAMINAE

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THE formation of chloroplasts has been dealt with recently in papers by Strugger (4–6), Heitz and Maly (1), and Leyon (2 and 3). Strugger found that the earliest stages of formation of chloroplasts consist of a colourless stroma enveloping a dense "primary granum" of the shape of a disc which stains readily with several dyes and contains the chlorophyll in leaves which are just turning green. Originally Strugger considered this "primary granum" itself as a hereditary unit multiplying through serial duplications and reduplications thus forming the stacked lamellae of the grana of the phanerogam plastid (4). This latter view has been opposed by Heitz and Maly (1) and by Leyon (2). In Strugger's most recent papers (5 and 6) his views appear to be somewhat modified since not the "primary granum" itself but each of its presumed elementary lamellae is identified with one single lamella of a granum-stack in the fullgrown plastid.

The significance of the "primary granum" as a cell constituent *sui generis* with identical reproduction is stressed, however, also in these latter reports by Strugger in which the "primary granum" is further described as the original site of the chlorophyll.

My earlier reports confirm the existence of a dense core in very young plastids during some stages of their development (2 Fig. 7 and 3, Fig. 3, 4 and 5). The first discernable laminae were described as "folds" originating from a layer in the young plastid near its dense core. For a long time I had not been able to find any connection between the core and the laminar system of the plastid.

However, the micrographs published in this report (Figs. 1–3) show that in somewhat later stages of the development of plastids, when new laminae are formed within each "fold", these laminae appear as extensions of distinct layers within the core. Furthermore the core (i.e. the primary granum according to Strugger) consists of regularly spaced material and thus appears as a crystal with a plane-spacing of the order of 25 mμ as measured from the micrographs.[1]

Material and methods. The techniques used were those discribed in an earlier report (3). The micrographs were selected from several similar pictures obtained from a young green shoot of *Aspidistra elatior*, about 5 cm high which was fixed and embedded in June. The investigated part was taken 1 mm from the top of the shoot.

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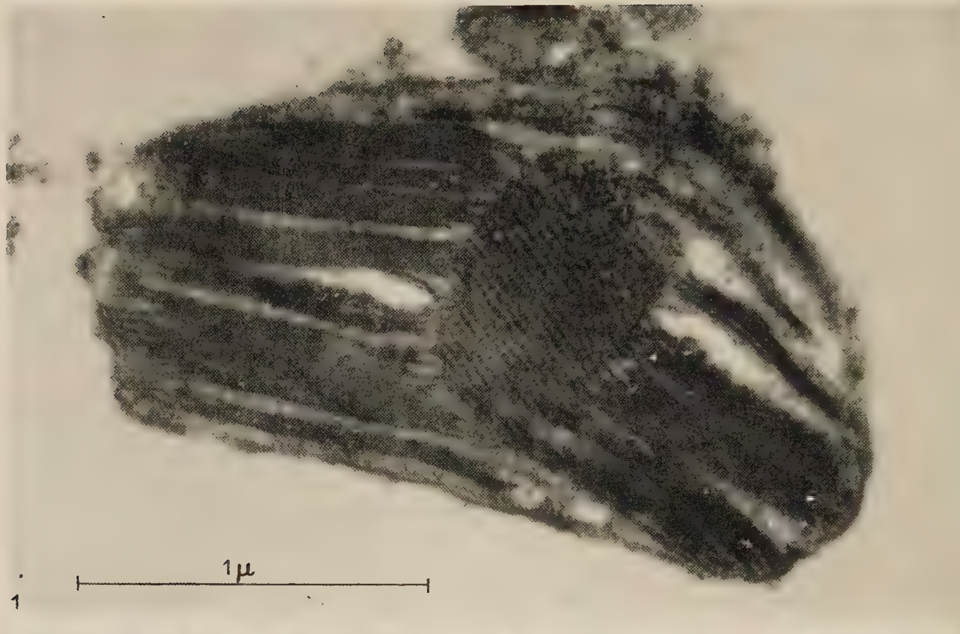
[1] According to Strugger (5) Böing has shown that the primary granum is double-refractive in one direction.



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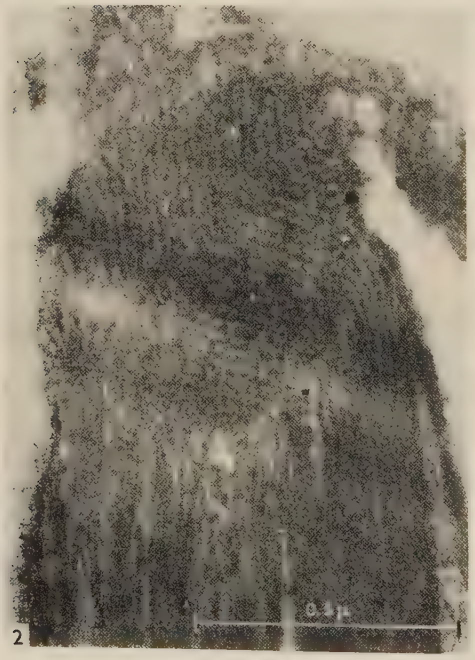

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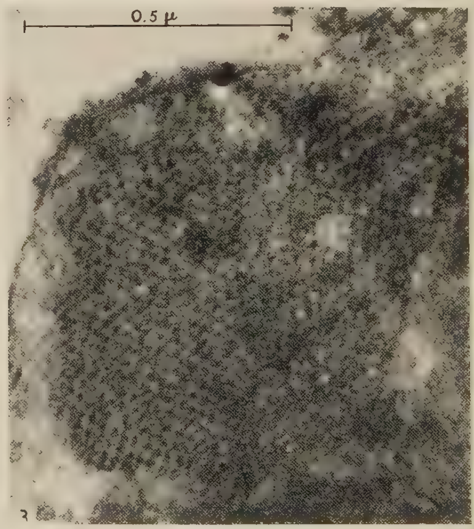

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This study was financially supported from Svenska Sockerfabriksaktiebolaget. To Professor Arne Tiselius my thanks are due for his helpful interest.

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Fig. 1. Young chloroplast of *Aspidistra elatior* with a dense core and groups of laminae.
Fig. 2. Part of a young chloroplast of *Aspidistra elatior* showing the connection between the chloroplast laminae and the layers of the dense core.
Fig. 3. The crystalline part of a young chloroplast of *Aspidistra elatior*.

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